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reference to the genes associated with ethylene production**

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**Akihiro Itai**

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## Introduction

Japanese pear is a fruit which has been cultivated in Japan since the Asuka era (600 A.D.) and is endemic to Japan, China and Korea. Early plantings of Japanese pear were little more than cultivated groves of the wild species (Kajiura, 1994). By the end of the Edo era, more than a thousand named cultivars were recorded. From the end of the Edo era to the Meiji era, commercial cultivation began in various parts of Japan. Commercial production made great strides since two important cultivars, 'Nijisseiki' and 'Chojuro', which were introduced in 1895, represented a major step forward in productivity and fruit quality. The flesh of 'Nijisseiki' is very juicy and crisp textured, while 'Chojuro' is sweet and disease resistant. These two cultivars occupied over 80% of pear production until the release of the cultivar 'Kosui', which has now become a major cultivar in Japan. Even now, these three leading cultivars 'Kosui', 'Hosui' and 'Nijisseiki' occupy over 80% of pear production. However, new cultivars with wider variation are awaited to reduce labor intensity especially the overlapping short harvest period.

In a breeding program of Japanese pear trees, improvement of fruit quality is a major objective (Abe et al., 1993; Machida and Kozaki; 1976). A few reports on the inheritance of fruit characters have dealt with Japanese pear and European pear (Abe et al., 1993; Abe et al., 1995; Crane and Lewis, 1949; Machida and Kozaki, 1975; Machida and Kozaki; 1976). It is important, for

increasing breeding efficiency, to elucidate the mode of inheritance of the main characters which influence fruit quality. These characters are the fruit weight, flesh firmness, soluble solid content, organic acid content, ripening time and storage potential. Although refrigeration systems have been developed, cultivation of cultivars with short storage potential is still limited and cultivars having long storage potential are predominant. Therefore, the storage potential is an important breeding objective in Japanese pear. With regard to fruit ripening characters in existing cultivars, there are wide genetic variations in the harvest season and storage potential.

It is generally thought that ethylene production affects ripening processes and shelf life in many fruits. In Japanese pear both climacteric and non-climacteric cultivars exist (Kitamura et al., 1981; Tanabe et al., 1994). Kitamura et al. (1981) reported that climacteric type fruits ( 'Kikusui' and 'Yakumo' ) showed a rise in respiration and ethylene production and that non-climacteric type fruits ( 'Nijisseiki' and 'Niitaka' ) did not show a rise in respiration and ethylene production. Tanabe et al. (1994) also reported that cultivars, such as 'Shinsui' , 'Kosui' and 'Chojuro' , exhibited a rapid increase in ethylene production and had a low storage potential and that in contrast, cultivars such as 'Nijisseiki' , 'Shinko' and 'Okusankichi' , showed non-detectable levels of ethylene and fruit quality kept for periods over a month. Moreover, ethephon, which is an ethylene releasing chemical is used practically to accelerate ripening time in Japanese pear production. The earlier harvested fruit has a higher commercial value and the orchard

scale can be increased owing to the dispersion of labor. Therefore, the storage potential is closely related to ethylene production in Japanese pear fruit.

In recent years, significant progress has been made in our understanding of the molecular aspects of fruit ripening, including the genetic control of temporal events during the ripening phase (Gillaspy et al., 1993; Seymour et al., 1993; Lelievre et al., 1997a) and it has become apparent that ripening, like other plant senescence processes, is under strict genetic control. Especially, considerable progress has been made in the characterization of ethylene biosynthetic genes (Nakajima et al., 1990; Lay-Yee and Knighton, 1995; Sato and Theologis, 1989; Theologis, 1992; Van Der Straeten et al., 1990) and ethylene signal transduction pathways (Bleecker and Schaller, 1996; Chang, 1996; Ecker, 1995).

It is well documented that ethylene is produced from methionine via S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylate (ACC) ( $\text{Met} \rightarrow \text{SAM} \rightarrow \text{ACC} \rightarrow \text{ethylene}$ ) and that three enzymes mediate individual steps: SAM synthase, ACC synthase and ACC oxidase (Kende, 1989; Yang and Hoffman, 1984). Most of the research on the molecular control of the pathway has been focused on the final two enzymes. Recently, cDNAs and genomic clones encoding these two enzymes have been isolated from various plant species (Dong et al., 1991; Holdsworth et al., 1987; Lay-Yee and Knighton, 1995; Liang et al., 1992; McGarvey et al., 1991; Nakajima et al., 1990; Sato and Theologis, 1989; Van der Straeten et al., 1990). The recent characterization and cloning of several ACC synthases and ACC oxidases has



given a tremendous impetus to investigations, at the molecular level, of the regulatory mechanisms that control the biosynthesis of ethylene. ACC synthase and ACC oxidase are encoded by a highly divergent multigene family whose members are differentially regulated by many factors such as abiotic stresses, mechanical strain, wounding, hypoxia, flooding, chilling, soil salinity and pathogen attack (Dong et al., 1991; Nakajima et al., 1990; Oetiker et al., 1997; Olson et al., 1995; Spanu et al., 1993; Van der Straeten et al., 1990). For example, in the tomato, six members of the ACC synthase multigene family are known (Oetiker et al., 1997; Nakatsuka et al., 1998). Four of them (*LE-ACS1A*, *LE-ACS2*, *LE-ACS3* and *LE-ACS4*) were expressed during fruit ripening and two of them (*LE-ACS2* and *LE-ACS4*) showed ripening-specific and wounding-induced expression (Nakatsuka et al., 1998). The importance of ethylene in ripening is evident from its stimulating effect on yellowing, softening, and ethylene production. Its role has been confirmed by showing that inhibitors of ethylene synthesis and action delay ripening. Moreover, the manipulation of ethylene in fruit via transgenic technology led to our understanding the role of ethylene in fruit ripening (Hamilton et al., 1990; Oeller et al., 1991).

It is thought that fruit ripening of Japanese pear is closely related to ethylene production. To permit selection of varieties having good storage potential in Japanese pear, a better understanding of the genetics of ethylene biosynthetic pathways is needed. While molecular events during ripening fruits have not been elucidated in detail, molecular cloning of specific genes involved in fruit ripening of Japanese pear may lead to a better

understanding of the determinants for fruit keeping, allowing the possibility of genetic engineering to impact on shelf life.

From this viewpoint, the objectives of my study were to clarify the following:

- 1) Ethylene production during fruit ripening and storage potential in existing cultivars .
- 2) The mode of inheritance of ethylene production during fruit ripening.
- 3) The development of the gene diagnosis of ethylene production during fruit ripening.
- 4) Isolation of genes related to fruit ripening and possible involvement of ethylene in their expression.

Information gained from this study should be useful for developing breeding strategies to improve the storage potential in Japanese pear fruit.

## **Chapter 1.**

# **Cultivar differences in the ethylene production during fruit ripening**

### **Introduction**

There are many types of Japanese pear fruits differing in shape, peel color, sugar content and ripening characteristics (Kajiura and Sato, 1990). According to ripening characteristics, the fruit can be classified as climacteric or non-climacteric. Climacteric fruits are characterized by the increase in ethylene production and respiration rate at the onset of ripening, whereas non-climacteric fruits do not exhibit these characteristics (Biale and Young, 1981). In Japanese pear, some cultivars produce appreciable amounts of ethylene, while others produce a small amount of ethylene (Kitamura et al., 1981; Tanabe et al., 1994). Kitamura et al. (1981) reported that climacteric type fruits ( 'Kikusui' and 'Yakumo' ) showed a rise in respiration and ethylene production, while non-climacteric type fruits ( 'Nijisseiki' and 'Niitaka' ) did not. Tanabe et al. (1994) also reported that cultivars, such as 'Shinsui' , 'Kosui' and 'Chojuro' , exhibited a rapid increase in ethylene production and that in contrast, cultivars such as 'Nijisseiki' , 'Shinko' and 'Okusankichi' , showed non-detectable levels of ethylene. However, further studies on ethylene production in Japanese cultivars have not been conducted. Available information on ethylene production is limited to a few

cultivars. This chapter describes the ethylene production during ripening in fruit of many existing cultivars I measured in an effort to clarify the ripening characteristics of Japanese pear and to improve breeding and post harvest handling information.

## **Materials and methods**

### *Plant materials*

Fifty-nine cultivars of Japanese pear (*Pyrus pyrifolia* Nakai) grown at the orchard of Tottori University were used (Table 1.1). They were classified into three groups according to Kajiura and Sato (1990).

- 1: Cultivars cultivated in the Edo era
- 2: Cultivars released from Meiji era to 1945 A.D.
- 3: Cultivars released after 1945 A.D.

### *Ethylene measurement during fruit ripening*

Three to five pear fruits of each cultivar were harvested during the fruit ripening season from the orchard of Tottori University and immediately placed in 1.5L sealed jars for 2 hr at 20°C for ethylene measurement. After 2hr, 2.0mL headspace gas samples were drawn from each jar. Samples were analyzed using gas chromatography, with a flame ionization detector and 60/80 mesh activated alumina column (Model 163, Hitachi). Ethylene measurements were obtained every 3-4 days for 2 weeks during fruit ripening and the maximum ethylene level of each fruit was recorded. The ethylene

Table 1.1 Cultivars used in this study

Cultivars cultivated in Edo era (<1867A.D.)	Cultivars released from Meiji era to 1945 A.D.	Cultivars released after 1945 A.D.
Awayuki	Akaho	Echigonishiki
Edoya	Amanokawa	Hosui
Hatsushimo	Asahiryu	Kosui
Imamuraaki	Atago	Kumoi
Imamuranatsu	Chojuro	Shinsei
Inugoroshi	Doitsu	Shinsetsu
Konpeito	Gion	Shinsui
Koyuki	Gozennashi	Suisei
Okuroku	Hakuteiryu	Suishu
Okusankichi	Hattatsu	Tama
Rokugatsu	Hokkaiwase	
Ruisannashi	Ichiharawase	
Shirayuki	Ishiiwase	
Sotoorihime	Kikusui	
Waseaka	Kimitsukawase	
Yokogoshi	Kinchaku	
	Kozo	
	Niitaka	
	Nijisseiki	
	Ninomiya	
	Ninomiyahakuri	
	Ohiromaru	
	Rikiya	
	Sekaiichi	
	Seigyoku	
	Seiryu	
	Shinchu	
	Shinko	
	Taihaku	
	Tosanishiki	
	Wasekozo	
	Yakumo	
	Yanaga	

measurements were carried out in 1993-1995 and 1997. The results of 1993-1995 and 1997 are shown in Fig. 1.1 and Table 1.2, respectively.

## Results and discussion

None of the cultivars showed much ethylene production until they reached the optimum harvest time. Then, the pattern of ethylene increase was cultivar dependent. 'Ninomiya', 'Rokugatsu', 'Edoya', 'Okuroku', 'Awayuki', and 'Sotoorihime' pears produced more than  $50 \mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr}$  (Fig. 1.1, Table 1.2), with 'Edoya' pear peaking at over  $300 \mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr}$ . These cultivars are known to have a poor storage potential (Kajiura and Sato, 1990). 'Shinsui', 'Kosui', 'Chojuro', 'Yakumo', 'Kikusui' and further 12 cultivars produced ethylene at moderate levels ( $0.5 \sim 10 \mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr}$ ) (Fig. 1.1) and are also known to have a short shelf life (Kajiura and Sato, 1990). 'Nijisseiki', 'Hosui', 'Niitaka', 'Okusankichi' and further 8 cultivars produced very little ethylene (less than  $0.5 \mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr}$ ) (Fig. 1.1) and had a long storage potential. The amount of ethylene produced was less than the level of lower limit ( $0.1 \mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr}$ ) detected by gas chromatography in almost all of these cultivars. Every Japanese pear cultivar showed a characteristic pattern of ethylene evolution, some having a rapid increase in ethylene production and others having no or very little ethylene production. The maximum rate of

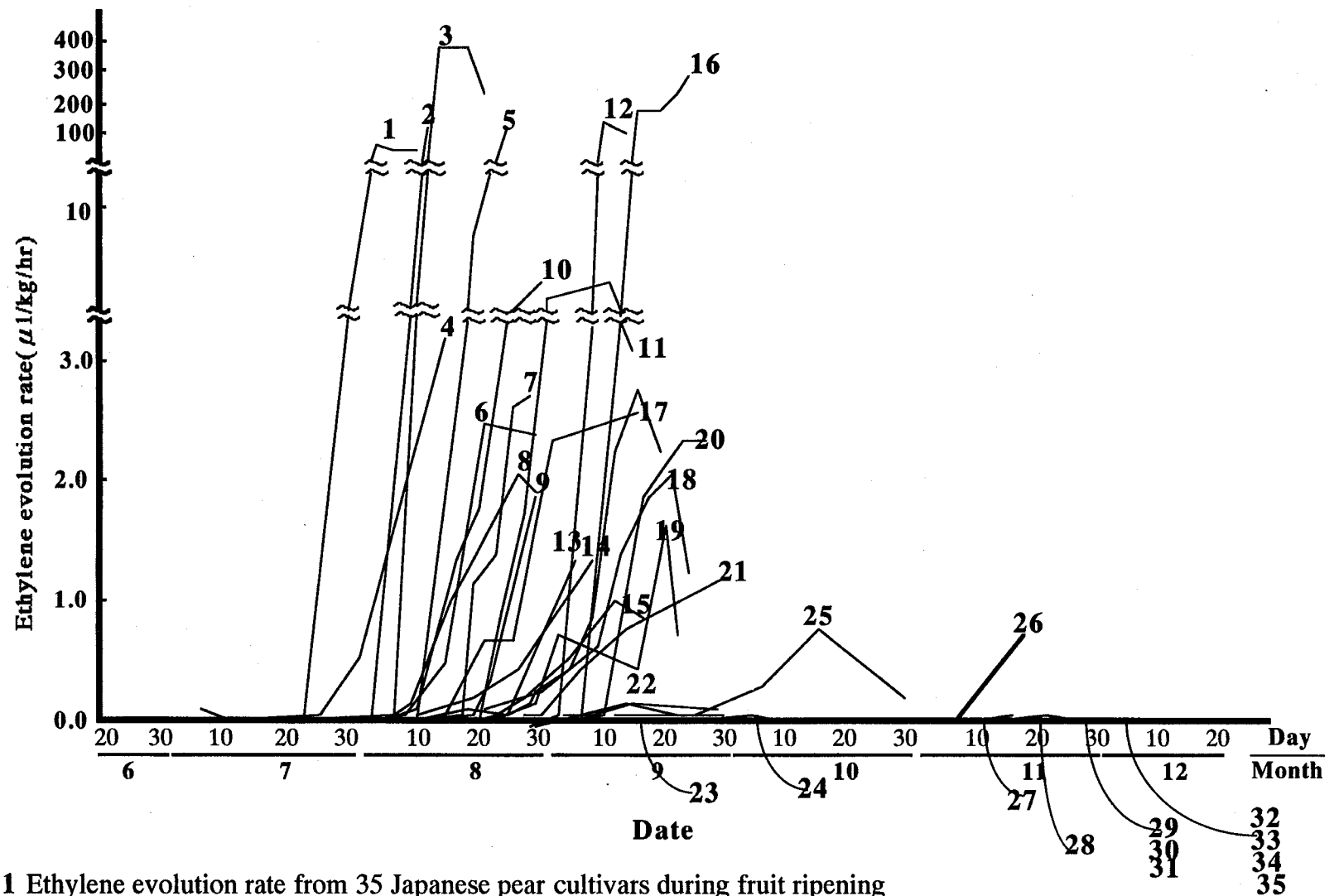


Fig. 1.1 Ethylene evolution rate from 35 Japanese pear cultivars during fruit ripening

1:Ninomiya 2:Rokugatsu 3:Edoya 4:Kimitsukawase 5:Okuroku 6:Ishiiwase 7:Suisei 8:Akaho 9:Shinsui  
 10:Shinchu 11:Kinchaku 12:Awayuki 13:Yakumo 14:Kosui 15:Doitsu 16:Sotoorihime 17:Wasekozo 18:Kozo  
 19:Gion 20:Chojuro 21:Kikusui 22:Nijisseiki 23:Sekaiichi 24:Hosui 25:Ohiromaru 26:Inugoroshi 27:Niitaka  
 28:Konpeito 29:Waseaka 30:Yokogoshi 31:Ruisannashi 32:Okusankichi 33:Imamuraaki 34:Amanokawa 35:  
 Shinsetsu

Table 1.2 Classification of cultivars based on ethylene production in ripening fruits

Maximum ethylene production during fruit ripening	Cultivars (Ethylene level: $\mu\text{l/kg/hr}$ ) <sup>a</sup>
High ( $>10\mu\text{l/kg/hr}$ )	Awayuki (105.8), Cili ( <i>P.bretschneideri</i> )(48.5), Edoya (302.1), Gozennashi (23.7), Hattatsu (32.2), Ninomiya (76.1), Ninomiyahakuri (43.3), Okuroku (135), Rikiya (170.8), Rokugatsu (55.4), Sotoorihime (192.5), Yali ( <i>P.bretschneideri</i> )(57.3)
Moderate ( $0.5\mu\text{l/kg/hr}$ - $10\mu\text{l/kg/hr}$ )	Akaho (2.18), Asahiryu (2.06), Chojuro (2.29), Doitsu (1.18), Gion (1.12), Hakuteiryu (1.01), Hokkaiwase (1.55), Ichiharawase (1.40), Imamuranatsu (1.67), Inugoroshi (0.98), Ishiiwase (6.19), Kikusui (1.18), Kimitsukawase (3.09), Kinchaku (2.77), Kosui (1.38), Kozo (0.94), Kumoi (2.85), Ohiromaru (0.94), Seigyoku (0.73), Shinchu (2.20), Shinsei (2.40), Shinsui (1.55), Suisei (0.93), Suishu (2.99), Taihaku (0.96), Tama (2.09), Wasekoko (0.98), Yakumo (0.51)
Low ( $< 0.5\mu\text{l/kg/hr}$ )	Amanokawa (0.04), Atago (0.10), Echigonishiki (0.04), Hatsushimo (0.06), Hongli ( <i>P.bretschneideri</i> )(0.03), Hosui (0.07), Imamuraaki (0.04), Konpeito (0.05), Koyuki (0.04), Niitaka (0.04), Nijisseiki (0.04), Okusankichi (0.02), Ruisannashi (0.05), Seiryu (0.15), Sekaiichi (0.20), Shinko (0.02), Shinsetsu (0.01), Shirayuki (0.03), Tosanishiki (0.20), Waseaka (0.13), Yanaga (0.03), Yokogoshi (0.08)

<sup>a</sup>Ethylene level was measured using 3 to 5 ripening fruits of each cultivars in 1997.



ethylene production ranged from 0.1 to 300  $\mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr.}$  during ripening of Japanese pear cultivars. In general, earlier maturing cultivars produce more ethylene than later maturing ones (Abeles et al, 1992) and a similar observation has been obtained for apple cultivars (Hansen, 1945). In a study of eleven apple cultivars, internal ethylene levels were around 0.1  $\mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr}$  until they reached the optimal harvest date and at that point the rate of ethylene production increased drastically (about 1000-fold) for some cultivars such as 'McIntosh', 'Spartan', 'Northern Spy', 'Golden Delicious', and 'Mutsu', moderately (about 100-fold) for 'Empire' and 'Delicious', or slightly (about 10-fold) for 'Jonagold' and 'Idared' (Chu, 1984). These results are similar to those observed in this study.

In the Edo era, high ethylene producers such as 'Rokugatsu', 'Edoya', 'Okuroku' and 'Sotoorihime' and low ethylene producers such as 'Imamuraaki', 'Yokogoshi', 'Okusankichi' and 'Waseaka' were cultivated and utilized. Thus, during this era, cultivars were classified into two major groups and low ethylene producers were predominant because of their storage ability. Thereafter, as compared with cultivars in the Edo era, cultivars released from the Meiji era to 1945 A.D. showed a higher percentage of moderate ethylene producers and lower percentage of high ethylene producers. Later, newly bred cultivars were classified into either moderate ethylene producers or low ethylene producers. Thus, there tended to be an increase of moderate ethylene producers, decrease of low ethylene

producers and scarcity of high ethylene producers over the years, which may have resulted in a narrow genetic variation. These findings are partially in accordance with those reported by Kajiura (1981).

The relationship between harvest season and ethylene level during fruit ripening is shown in Table 1.3. Early-maturing cultivars were classified into either high or moderate ethylene producers without exception. Low ethylene producers were not included in early-maturing cultivars. In mid-maturing cultivars, all types of ethylene producers were included, while all of the late-maturing cultivars were classified into low ethylene producers with the exception of 'Ohiromaru' and 'Inugoroshi'. High ethylene producers were not found in late-maturing cultivars. High and moderate ethylene producers tended to be early- or mid-maturing cultivars and low ethylene producers tended to be mid- or late-maturing cultivars. These results suggest that harvest season is closely related to the maximum ethylene level during fruit ripening. Generally, late-maturing cultivars had good storage potential and were used for long storage (Kajiura, 1981).

These findings demonstrate that harvest season, storage potential and ethylene level during fruit ripening are related to one another. Furthermore, these data show that the characteristics of ethylene production are selective indicators of harvest season and storage potential.

Table 1.3 Relationship between ethylene production and harvest season<sup>a</sup> in Japanese pear fruit

Early-maturing cultivars (maximum ethylene level <sup>b</sup> )	Mid-maturing cultivars (maximum ethylene level)	Late-maturing cultivars (maximum ethylene level)
Akaho (M)	Awayuki (H)	Amanokawa (L)
Edoya (H)	Asahiryu (M)	Atago (L)
Ichiharawase (M)	Chojuro (M)	Echigonishiki (L)
Ishiiwase (M)	Doitsu (M)	Hatsushimo (L)
Kimitsukawase (M)	Gion (M)	Imamuraaki (L)
Kosui (M)	Gozennashi (H)	Inugoroshi (M)
Kumoi (M)	Hakuteiryu (M)	Konpeito (L)
Ninomiya (H)	Hattatsu (H)	Koyuki (L)
Okuroku (H)	Hokkaiwase (M)	Niitaka (L)
Rokugatsu (H)	Hosui (L)	Ohiromaru (M)
Shinchu (M)	Imamuranatsu (M)	Okusankichi (L)
Shinsui (M)	Kikusui (M)	Ruisannashi (L)
Suishu (M)	Kinchaku (M)	Shinko (L)
Tama (M)	Kozo (M)	Shinsetsu (L)
Yakumo (M)	Nijisseiki (L)	Shirayuki (L)
	Ninomiyahakuri (L)	Tosanishiki (L)
	Rikiya (H)	Waseaka (L)
	Sekaiichi (L)	Yanaga (L)
	Seigyoku (M)	Yokogoshi (L)
	Seiryu (L)	
	Shinsei (M)	
	Sotoorihime (H)	
	Suisei (M)	
	Taihaku (M)	
	Wasekoso (M)	

<sup>a</sup>Harvest season is classified into 3 categories.

Early: ~August, Mid: September, Late: October~

<sup>b</sup>Maximum ethylene level during fruit ripening

H:  $>10\mu\text{L/kg/hr}$ , M:  $0.5\mu\text{L/kg/hr}\sim 10\mu\text{L/kg/hr}$ , L:  $<0.5\mu\text{L/kg/hr}$

## **Chapter 2.**

### **Isolation and expression analysis of ethylene biosynthetic genes**

#### **2.1 Isolation of cDNAs encoding for 1-aminocyclopropane-1-carboxylate synthase from ripening fruits and differences in the gene expression during fruit ripening with the cultivar**

##### **Introduction**

As described in the previous chapter, the ethylene level in Japanese pear fruit during ripening varied with the cultivar from 0.1 to 300  $\mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr.}$  Ethylene is synthesized from SAM via ACC (Yang and Hoffman, 1984; Kende, 1993). The first step in ethylene production is catalyzed by ACC synthase. The cloning and the characterization of genes encoding ACC synthase has been done in many species (Nakajima et al., 1990; Lay-Yee and Knighton, 1995; Sato and Theologis, 1989; Van Der Straeten et al., 1990). ACC synthase is encoded by a multigene family and that its various members are differentially expressed in response to many factors (Lincoln et al., 1993, Olson et al., 1991, Rottmann et al., 1991). Generally, the rate-limiting step in ethylene production has been considered to be ACC synthase (Theologis, 1992). In most cases, increase in ethylene production is due to enhanced transcription of ACC synthase genes (Kende, 1993). However, recent studies

have shown that regulatory mechanisms at the post-transcriptional and post-translational level are equally important in ethylene production (Peck and Kende, 1998; Spanu et al., 1993).

Since ACC synthase plays a key role in ethylene biosynthetic pathway, I examined its regulation at the molecular level, in relation to fruit ripening in Japanese pear. Here I report the isolation and expression analysis of cDNAs encoding ACC synthase which is correlated with ethylene production in the Japanese pear during ripening.

## **Materials and methods**

### *Plant materials*

Eleven cultivars of Japanese pear grown at the orchard of Tottori University were used in this study. For gene cloning cvs 'Chojuro' , 'Nijisseiki' and 'Ninomiya' were selected and for gene expression analysis, fruits from 11 cultivars ( 'Ninomiyahakuri' , 'Ninomiya' , 'Edoya' , 'Rokugatsu' , 'Awayuki' , 'Shinsui' , 'Kikusui' , 'Nijisseiki' , 'Chojuro' , 'Shinsetsu' and 'Hosui' ) were used.

### *RNA extraction and RT-PCR*

Total RNA was extracted by the hot borate method (Wan and Wilkins, 1994). cDNA was synthesized using the AMV Reverse Transcriptase First-strand cDNA synthesis kit (Life Science, USA) using 5 $\mu$ g of total RNA from

ripe 'Ninomiya', 'Nijisseiki' and 'Kikusui' Japanese pear fruit, followed by PCR with appropriate primers. Oligonucleotide primers for ACC synthase were designed as follows: For PPACS1, primers based on the pear sequence (cv. Passe-Crassane), accession number: X87097 (Lelievre et al., 1997b) were used. Upstream primer was 5'-ACCTCGAACTCTCTCTAATGC-3' and downstream primer was 5'-AATGCATCATAATCACAATGGAG-3'. For PPACS2 and PPACS3, degenerate primers based on conserved regions of ACC synthases in the Genbank database were used. Upstream primer was 5'-ATICARATGGGIYTIGCIGARAAYCA-3' and downstream primer was 5'-GCRAARCAIACICKRAACCAIC-CIGGYTC-3'. PCR conditions for PPACS1 were 40 cycles of 94°C for 1min, 62°C for 1min and 72°C for 2min. Conditions for PPACS2 and PPACS3 were the same except for annealing at 57°C and 45°C, respectively.

Amplified fragments (1.8kb for PPACS1 from 'Ninomiya' fruit cDNA, 1.1kb for PPACS2 from 'Kikusui' fruit cDNA and 1.1 kb for PPACS3 from 'Nijisseiki' fruit cDNA) were purified by gel electrophoresis and ligated into the pGEM-T vector (Promega). The TA-cloned cDNA insert was partially sequenced using the ALF Express Automatic Sequencer (Pharmacia) with the Thermo-Sequenase Fluorescent Sequencing Kit (Amersham). Since the partial sequence of cloned insert had high homology with ACC synthase genes from other plant species, the cloned insert was used as a library screening probe.

### *Construction and screening of cDNA library*

Poly(A)<sup>+</sup> RNA was isolated using oligo-dT columns (Pharmacia) from total RNA of 'Kikusui' and 'Nijisseiki' fruit extracted as described above. Oligo-dT primed cDNA was cloned in lambda Excell (Pharmacia) and cDNA libraries of 120,000 clones from 'Kikusui' and 200,000 clones from 'Nijisseiki' were constructed. For screening, plaques were transferred to Hybond N<sup>+</sup> nylon membranes (Amersham) and hybridized in 40% formamide, 5xSSPE, 5xDenhardt's, and 0.1% SDS at 42°C, with the appropriate probe. Following hybridization, the filters were washed at 65°C in 0.2 x SSC, and 0.1% SDS. The cloned 1.1kb fragment of *PPACS2* and *PPACS3* were <sup>32</sup>P-labeled by the random primed method (Rediprime kit, Amersham) and used in hybridization as described above. The 'Kikusui' cDNA library was used for *PPACS2* gene screening and the 'Nijisseiki' cDNA library was used for *PPACS3* gene screening. Phagemids were released *in vitro* from positive plaques and DNA extracted by the Alkaline-SDS method (Sambrook et al., 1989).

### *Cloning of ACC synthase cDNAs*

Using the *PPACS2* fragment as a probe to the 'Kikusui' cDNA library, 6 positive clones were detected. The longest cDNA insert, *PPACS2*, was sequenced using the Thermo-sequenase fluorescent sequencing kit (Amersham) with an ALF express sequencer (Pharmacia). Using the *PPACS3*

fragment as a probe to the 'Nijisseiki' pear cDNA library, 2 positive clones were detected. The longest cDNA insert, *PPACS3*, was sequenced as above. The TA cloned cDNA insert, *PPACS1* was also sequenced. Nucleotide sequences were analyzed using DNASIS software (Hitachi).

#### *DNA isolation and Southern blot analysis*

Total DNA was extracted from the immature leaves of 'Nijisseiki' by the modified SDS method (Teramoto et al., 1994). Ten  $\mu$ g aliquots of the DNA were digested with *Eco*RI or *Hind* III, separated by electrophoresis in 0.9% agarose gels, and transferred to Hybond N<sup>+</sup> nylon membranes (Amersham). Hybridization of the DNA blots was performed as for library screening. Post-hybridization washes were 2 x 15 minutes at 65°C in 0.2 x SSC and 0.1% SDS, after which the membranes were exposed to an Imaging plate (Fuji Film, Tokyo, Japan). Signals were detected with an image analyzer (FLA2000; Fuji Film).

#### *Northern blot and RT-PCR analysis*

To analyze the cultivar differences in gene expression, the fruit at the ripening stages of 11 cultivars ( 'Ninomiyahakuri' , 'Ninomiya' , 'Edoya' , 'Rokugatsu' , 'Awayuki' , 'Shinsui' , 'Kikusui' , 'Nijisseiki' , 'Chojuro' , 'Shinsetsu' and 'Hosui' ), which show various maximum ethylene production levels during fruit ripening, were collected. For gene



expression analysis, total RNA was extracted from these samples as described above. 'Nijisseiki', 'Hosui' and 'Shinsetsu' are low ethylene producers ( $\leq 0.5 \mu\text{l/kg f.w./hr.}$ ), 'Kikusui', 'Chojuro' and 'Shinsui' are moderate ethylene producers ( $0.5 \mu\text{l/kg f.w./hr} \sim 10 \mu\text{l/kg f.w./hr}$ ), and the remaining 5 cultivars are high ethylene producers ( $\geq 10 \mu\text{l/kg f.w./hr}$ ). To study the effect of wounding on the expression of ACC synthase genes, approximately 10 g of flesh from 'Nijisseiki' overripe fruit (23DAH: Days after harvest; harvest date is 153 DAF: Days after full bloom ) was cut into pieces and held in an open flask at 20 °C for 24 hr. The cut samples were harvested at 0, 6, 12, and 24 hr after wounding treatment for RNA extraction. For Northern analysis, 10  $\mu\text{g}$  of total RNA was fractionated on a 1.2% agarose gel containing formaldehyde and blotted onto Hybond N+ nylon membranes (Amersham). Membranes were hybridized with  $^{32}\text{P}$ -labeled *PPACS1*, *PPACS2* and *PPACS3* cDNA fragments.

For RT-PCR analysis, first strand cDNA was synthesized using the AMV reverse transcriptase first-strand cDNA synthesis kit from 3  $\mu\text{g}$  of total RNA used for Northern analysis. Oligonucleotide primers specific for three ACC synthase genes were designed as follows. For *PPACS1*, ACS1-1 (5'-ACCTCGAACTCTCTCTCTAATGC-3') and ACS1-2 (5'-AATGCATCATAATCACAATGGAG-3'), for *PPACS2*, ACS2-1 (5'-GTCACAGAATCAACGATTGA-3') and ACS2-2 (5'-AGTAGAACGCCAAAACAAAT-3'), and for *PPACS3*, ACS3-1 (5'-

CTACTGTCGTTCCAGACTTCCAGTA-3') and ACS3-2 (5'-TGATACAATGGGGAGTCCTACAAGC-3'). PCR conditions for *PPACS1*, *PPACS2* and *PPACS3* were the same 40 cycles of 94°C for 1min, 62°C for 1min and 72°C for 2 min, with a final extension of 5 min at 72°C. After PCR reaction, PCR mixture was run on 0.9% agarose gel and DNA bands were detected by ethidium bromide staining.

## Results

### *Isolation of cDNAs for ACC synthase*

*PPACS1* cDNA was amplified by RT-PCR using primers designed for the nucleotide sequence of pear cv 'Passe-Crassane' (pPC-ACS1). The cDNA (*PPACS1*) insert was 1778 bp in length, and contained an open reading frame of 1419 bp (Fig. 2.1). This open reading frame coded for a protein of 473 amino acids. The predicted molecular mass of the protein encoded by *PPACS1* is 53.4 kD.

*PPACS1* had high homology (98%) to pPC-ACS1 (Table 2.1). *PPACS2* cDNA was 1872 bp in length, and contained an open reading frame of 1338 bp (Fig. 2.2). This open reading frame coded for a protein of 446 amino acids. The predicted molecular mass of the protein encoded by *PPACS1* is 45 kD. The coding region of *PPACS2* is 97% identical to MdACS-3 from ripening apple fruit (Rosenfield et al., 1996) (Table 2.2).

*PPACS3* cDNA insert was 1891 bp in length, and contained an open reading frame of 1485 bp (Fig. 2.3). This open reading frame coded for a

1	ACCTCGAACTCTCTCTATTGCTTT	26
27	CTCTTCCTTTCCAACTTCTGTCTTATAGCTTGATCCATACAGAAGAAAATTAACCAAA	86
87	ATGCGCATGTTATCCAGAAACGCTACGTTCAACTCTCACGGCCAAGACTCCTCCTACTTC	146
1	M R M L S R N A T F N S H G Q D S S Y F	20
147	TTAGGTTGGCAAGATGAGAAGAACCCTACCATGAGGTCCACAACACAACGGGATT	206
21	L G W Q E Y E K N P Y H E V H N T N G I	40
207	ATTCAGATGGGTCTCGCAGAAAATCAGCTCTGCTTTGATCTTCTCGAGTCATGGCTGGCT	266
41	I Q M G L A E N Q L C F D L L E S W L A	60
267	AAGAATCCAGAAGCAGCTGCATTTAAAAAATGGAGAATCCATATTTGCAGAGCTTGCT	326
61	K N P E A A A F K K N G E S I F A E L A	80
327	CTCTTCCAAGATTATCATGGCCTTCCCGCATTCAAAAAGCGATGGTAGATTTTCATGGCG	386
81	L F Q D Y H G L P A F K K A M V D F M A	100
387	GAAATCCGAGGGAACAAAGTGACCTTTGATCCCAACCACTTAGTGCTCACCGCCGGTGCA	446
101	E I R G N K V T F D P N H L V L T A G A	120
447	ACTTCAGCCAATGAGACCTTTATTTTCTGCCTTGCTGACCCCGCGAAGCGTTTCTTATT	506
121	T S A N E T F I F C L A D P G E A F L I	140
507	CCTACCCCATACTACCCAGGATTTGATAGAGACCTCAAGTGGCGAACTGGAGTCGAGATT	566
141	P T P Y Y P G F D R D L K W R T G V E I	160
567	GTACCCATTCACTGCACAACTCCAATGGCTTCCAATTACTGAAACCGCTCTGGAAGAA	626
161	V P I H C T N S N G F Q I T E T A L E E	180
627	GCCTACCAAGAAGCGGAAAAATGCAATCTCAGAGTCAAAGCGCTTGGTCACGAACCCA	686
181	A Y Q E A E K C N L R V K G V L V T N P	200
687	TCAAACCCATTGGGCACCACAATGACCAGAAACGAACCTCTACCTCCTCTTCTCCTCGTT	746
201	S N P L G T T M T R N E L Y L L L S F V	220
747	GAAGACAAAGGCATCCACCTCATTAGCGATGAAATTTACTCCGGCACGGCTTTTAGCTCC	806
221	E D K G I H L I S D E I Y S G T A F S S	240
807	CCATCCTTTATAAGCGTCATGGAAGTTCTCAAAGACAGAACTGTGATGAGAATTTCGAA	866
241	P S F I S V M E V L K D R N C D E N F E	260
867	GTTTGGCAGCGATTACGTTGTCTATAGCCTCTCTAAGGATCTTGGCCTTCCGGGTTTT	926
261	V W Q R V H V V Y S L S K D L G L P G F	280
927	CGGGTTGGCGCCATTTACTCCAACGACGACATGGTTGTGGCCGCCGCTACAAAAATGTCA	986
281	R V G A I Y S N D D M V V A A A T K M S	300
987	AGCTTTGGTCTTGTCTTCTCTCAAACCTCAGCACCTTCTCTCCGCCATGCTATCCGACAAG	1046
301	S F G L V S S Q T Q H L L S A M L S D K	320
1047	AAACTCACTAAGAATAACATAGCCGAGAACCACAAAAGACTCAAACAACGTACAGAAAAAT	1106
321	K L T K N Y I A E N H K R L K Q R Q K N	340
1107	CTCGTCTCCGGCCTTCAGAAAGCTGGCATTAGCTGCCTCAATGGCAATGCTGGCTTGTTTC	1166
341	L V S G L Q K A G I S C L N G N A G L F	360
1167	TGTTGGGTGGATATGAGGCACTTGCTTAGGTCAAACACCTTTGAAGCCGAAATGGAGCTC	1226
361	C W V D M R H L L R S N T F E A E M E L	380
1227	TGGA AAAAGATTGTATACGAAGTTCACCTCAATATCTCTCTGGATCGTCTTGTCATTGC	1286
381	W K K I V Y E V H L N I S P G S S C H C	400
1287	ACGGAACCTGGTTGGTTCGGTGTCTGCTTTGCCAACTTGCCGAGAGAACTCTGGACTTG	1346
401	T E P G W F R V C F A N L P E R T L D L	420
1347	GCAATGCAGAGACTGAAGGCATTTGTAGGGGAATATTACAACGTCCTGAGGTCAATGGC	1406
421	A M Q R L K A F V G E Y Y N V P E V N G	440
1407	CGCAGCCAAAGCAGCCATTTAAGCCACTCGAGAGGACAGTCGCTCACGAAGTGGGTTTCC	1466
441	R S Q S S H L S H S R G Q S L T K W V S	460
1467	CGGCTATCCTTCGATGACCGCTGTCTATTACGGTAGATGAAAGGTAGCCTGGTCTGAG	1526
461	R L S F D D R C P I H G	
1527	CACAAGAAACCGCTGAGTAAAATTACATTTTAGACCAAACATTTTTTCTGCCAAAAAGT	1586
1587	TAATTGGTTGAATTTTATTTTCGTTTTAGTTATTTTTTTCTCCGAATGTAGAGAAGTG	1646
1647	CACTCGGTCCGTGTTTGGGATGTGAAGTGGCTTTAGCTTCATTTGTAAAAGTATTTTCG	1706
1707	GTGATTGTGTGACAACAATTTTCTTTATGTCTTCGAAAACAAAATTACTCCATTGTGA	1766
1767	TTATGATGCATT	

Fig. 2.1 Nucleotide and deduced amino acid sequences of *PPACS1*.

1	ATTAAACTACTTTATGTATGTAAAAGGCCAGCTTTGATGTATATCCTGCCTATGTCACA	60
61	GAATCAACGATTGATTCTTCTCTTTTGTCTCAATATACAGAACAGGCAAGTTGAG	120
121	AGTACCTTTTTGTTTATTATTGTTTATGCCATTGCTCTCAAAGCCTTCTATATATG	180
181	AAAGTGGGTGATTTCAAAGGTTTTTCATTGCAACAATTCTCATTCAACTCTTTCTCTCT	240
241	CTATCTGAGTCACACCAACAGCTTTCTTGAAGGCCAAAGTGTTTACAAGAAACCTAGTT	300
301	ATGGCTATAGATATTGAGCAGCAGCAGCAGCCTTCTCCTGGGCTATCAAAAATAGCTGTT	360
1	M A I D I E Q Q Q Q P S P G L S K I A V	20
361	TCTGATACTCATGGAGAAGACTCTCCATACTTTGCAGGATGGAAAGCTTATGATGAAAAC	420
21	S D T H G E D S P Y F A G W K A Y D E N	40
421	CCTTATCATGAGTCAAGCAACCCATCTGGAGTCATACAATGGGACTTGGGAAAATCAA	480
41	P Y H E S S N P S G V I Q M G L A E N Q	60
481	GTTTCGTTTGATCTGTGGAAAAGCACTTGGAAAGAAATTGAGAAGCCTCCAACCTGGGGT	540
61	V S F D L L E K H L E E N S E A S N W G	80
541	TCAAAGGGATCAAAAGGAGTGTCTGGCTTTAGAGAAAATGCCTTATTTCAAGACTACCAT	600
81	S K G S K G V S G F R E N A L F Q D Y H	100
601	GGCCTTTTGTCTTTGAGAAAGGCAATGGCAAGTTTATGGAACAAATTAGAGGAGGAAGA	660
101	G L L S F R K A M A S F M E Q I R G G R	120
661	GCCAAATTTGACCTGCTAGGGTAGTCTTAACGGCAGGTGCAACTGCAGCAATGAGCTG	720
121	A K F D P A R V V L T A G A T A A N E L	140
721	TTGACTTTCATCATAGCTGATCCCGGTGATGCTTTGCTTGTCCAACCCCATATTATCCA	780
141	L T F I I A D P G D A L L V P T P Y Y P	160
781	GGGTTTGATAGAGATTTGAGGTGGAGGACTGGTGTGAACATTGTGCCAATTCAGTGTGAA	840
161	G F D R D L R W R T G V N I V P I H C E	180
841	AGCTCAAACTTCCAGATTACTCCTCAAGCTTTAGAAGCTGCATACAAAGAGGCAGAA	900
181	S S N N F Q I T P Q A L E A A Y K E A E	200
901	GCCAAGAACATGAGAGTGAGAGGGGTACTAATCACAAATCCATCAAAACCCACTTGGTGCA	960
201	A K N M R V R G V L I T N P S N P L G A	220
961	ACAATCCAAAGGGCAGTCTTGAAGAGATTCTTGATTTGCTACCCAAAAAACATCCAT	1020
221	T I Q R A V L E E I L D F V T Q K N I H	240
1021	CTTGCTCTGATGAAATCTACTCGGGATCTGCCTTCTCATCCTCGAATTCATTAGTGTG	1080
241	L V S D E I Y S G S A F S S S E F I S V	260
1081	GCAGAAATCTTGAAGACAGGCAGTACAAGGATGCAGAAAGAGTCCACATTGTTTATAGT	1140
261	A E I L E D R Q Y K D A E R V H I V Y S	280
1141	CTATCCAAAGATCTTGGCCTTCCAGGCTTTGAGTTGGCACTGTGTACTCTTCAATGAC	1200
281	L S K D L G L P G F R V G T V Y S F N D	300
1201	AAGGTTGTGACAACAGCAAGAAGATGTCTAGCTTACCTTGATATCTTCTGAAACACAA	1260
301	K V V T T A R R M S S F T L I S S E T Q	320
1261	CATCTCTTGGCTTCCATGTTATCTGACAAGGAATTCACGGGAACTACATAAGACAAAT	1320
321	H L L A S M L S D K E F T G N Y I K T N	340
1321	AGAGAGAGACTGAGGAGGAGATATGATATGATCGTCGAGGGTTGAAAAAATCGGGGATC	1380
341	R E R L R R R Y D M I V E G L K K S G I	360
1381	GAGTGTTTGAAAGGAAATGCTGGGTTGTTTGTGGATGAATTTAAGTCCATTTTGGAC	1440
361	E C L K G N A G L F C W M N L S P F L D	380
1441	GAACCAACAAGAGAATGTGAAGTGAAGTCTTTGGGATTCATGCTGCATGAAGTGAAGCTA	1500
381	E P T R E C E L T L W D S M L H E V K L	400
1501	AATATATCTCCAGGTTCTTCTTGTCTGCTGAGCCAGGCTGGTTTAGGGTGTGCTTT	1560
401	N I S P G S S C H C S E P G W F R V C F	420
1561	GCCAACATGAGTGAGCAGACACTTGGGATTGCATTAACAAGAATACATAATTTTCATGGAA	1620
421	A N M S E Q T L G I A L T R I H N F M E	440
1621	AAGAGAGAGCGGGCTTGCTAGAATAAAGTTTCTCTCTTTTATCTCTTTTTTTCAGTGG	1680
441	K R E R A C *	
1681	TGTAAGCCATTTTTTATTCTTTTGGAGGACAAAACCAATTTACTCTTTCTGAATTGTAA	1740
1741	CAACTCCATCTTGTCTTCTTGTCTTGAAGCAAATTTATACATTTCAGTTTGTATAATC	1800
1801	GATGGATATAATATTGTTTGGCGTTCTACTAAAAAATAAAAAAAAAAAAAAAAAAAAAA	1860
1861	AAAAAAAAAAAAA	

Fig. 2.2 Nucleotide and deduced amino acid sequences of *PPACS2*.

1	CTACTGTCGTTCCAGACTTCCAGTAGTATAATCATTCCCTCCAGCTATAT	50
51	AGCTTGTGTGTGTTCTTTGGAACCAAGAAAATGGCCTCATCTGCAAGTGAGAATCGCTTA	110
111	CTGCTATCCAAGATTGCAACCAATGAAAAACATGGTGAGAACTCGCCGTACTTCGACGGA	170
171	TGGAATAAGTACAATCAAAAACCATTTACCTAACTGAAAACTCTGAAGGAGTTATCCAG	230
231	ATGGGTCTGGCAGAAAATCAGCTTTCTTCGACTTGGTTGAAGAGTGGATTAGGAAAAAT	290
	1 M G L A E N Q L S F D L V E E W I R K N	20
291	CCCAAAGCCTCTATTTGCACTGCTGAAGGAATTGAGAAGTTCAGAAACGTGGCCAATTTT	350
21	P K A S I C T A E G I E K F R N V A N F	40
351	CAAGACTATCATGGCTTACCAGAGTTCAGACAGGCCATTGCTACGTTTCATGTCGAAAGCA	410
41	Q D Y H G L P E F R Q A I A T F M S K A	60
411	AGAGGTGGTAGAGTCACATTTGATCCTCATCGCGTAGTTATGAGTGGAGGAGCCACCGGA	470
61	R G G R V T F D P H R V V M S G G A T G	80
471	GCAATAGAGCTGGTCATGTTCTGTTTGGCCGACCGCGCGATGCTTTCCTTATCCCTCA	530
81	A N E L V M F C L A D R G D A F L I P S	100
531	CCGTAATATCCAGCATTTTACCGAGACCTCGGATGGAGAACTGGAGTCCAAATGTCCCG	590
101	P Y Y P A F Y R D L G W R T G V Q I V P	120
591	GTCGATTGTGATAGCTCAACAATTTCAAAATAACCAAGGAAGCACTGGAAGCAGCTTAT	650
121	V D C D S S N N F K I T K E A L E A A Y	140
651	GAAAAAGCCAAAACAACATCAATGTCAAGGGCTTGATCATAACAAACCATCAAAAT	710
141	E K A Q N N N I N V K G L I I T N P S N	160
711	CCATTAGGCACAACCCTAGACAAAGACACTTGAAGCCTTGTACATTTATAAACCAA	770
161	P L G T T L D K D T L E S L V T F I N Q	180
771	AAGAATTCACCTTGGTTTGTGATGAAATCTATGCAGCCACAGTCTTCAGCTCCCCAAA	830
181	K N I H L V C D E I Y A A T V F S S P K	200
831	TTACATGCATCACCAGGTTATACAAAACATGAATTGCAACCCCAACCTAATCCACATT	890
201	F T C I T E V I Q N M N C N P N L I H I	220
891	GTCTACAGTTTGTCCAAGGACATGGGGTTCCCGGACTGAGAGTGGCATCGTTTACTCC	950
221	V Y S L S K D M G F P G L R V G I V Y S	240
951	TACAACGATGACGTGGTGAACATTGGCCGAAAAATGTCAAGTTTGGGCTGGTCTCATCC	1010
241	Y N D D V V N I G R K M S S F G L V S S	260
1011	CAAACCTCAGCACATGCTTGCCTCATGCTTTCGGACGAAGATTTTGTGAGAAGTTCCTC	1070
261	Q T Q H M L A S M L S D E D F V E K F L	280
1071	ACCACAAGCGCAAAAAGGCTCGCAAGAGGCACGGGGTCTTCACTGATGGGCTTGAGGAA	1130
281	T T S A K R L A K R H G V F T D G L E E	300
1131	GTGGGAATCAACTGCTTGAAGAGCAATGCCGGCCTTTTCTGTTGGATGGACTTAAGGAGG	1190
301	V G I N C L K S N A G L F C W M D L R R	320
1191	CTATTGAAAGATCAACGTTTGTATGGCGAAATGGTGTGTGGCGTGAATCGTTAATGAA	1250
321	L L K D Q T F D G E M V L W R V I V N E	340
1251	GTGAAGCTCAACGTTTCTCCGGGCTCTTCTTTAAATGCGTTCGAGCCTGGTTGGTTAGG	1310
341	V K L N V S P G S S F K C V E P G W F R	360
1311	GTTTGTCTCGCCAACATGGATGATGACACAGTGAAGTTGCACTCAAAGGATTAGGGCA	1370
361	V C F A N M D D D T V E V A L K R I R A	380
1371	TTTGTAGGGCAAGGAAAGAAAGCTCAAGAACAAGCACAAGTGAAGTCTAAGAAGCGT	1430
381	F V G Q G K K A Q E Q A Q V K S P K K R	400
1431	TGGCAGAGCAATCTAAGGCTGAGCTTTTCATCATCATCCAGAAGGTTTGAGCAGGAAGGT	1490
401	W Q S N L R L S F S S S S R R F E Q E G	420
1491	GTTAGTGTATATGCACACGATGATGTCTCTCATTACCAATGCCTCACTCACCTCTC	1550
421	V S V M S P R M M S P H S P M P H S P L	440
1551	CTTCGAGCTACGACTTAAAGAGTATTGGGGACCTATACTCTAGTGTGTGCAATTAATTA	1610
441	L R A T T *	
1611	GGGTACTAGAAAATAATTACTGAGGTTGAATATGTTTTTAATTCGTTTTGGACGTGCTC	1670
1671	ATGGTAATTTTGCCATTATAATTACTTTGGATGCTGATTAATGCAGCTCTCAAAGGTTT	1730
1731	TTTTTAATGGACTAAATAGGAAAGCTGTCGTTCATTTTTCTTCAAATATCTATCAAT	1790
1791	AGCGTGAATAGTCATATTGCTAATCCTCTCTATTGAGGCCTTAATATGCTTGTAGGA	1850
1851	CTCCCCATTGTATCAAGCACTTTTTCTTAAAAGGAAAAA	

Fig. 2.3 Nucleotide and deduced amino acid sequences of PPACS3.

protein of 495 amino acids. The predicted molecular mass of the protein encoded by *PPACS3* is 55.5 kD. The predicted polypeptide showed 72% amino acid identity with an ACC synthase cDNA clone (p-GAC2) from *Pelargonium hortorum* (Wang and Arteca, 1995), 69% identity with a potato clone (STACS5) (Schlagnhauser et al., 1997), 68% identity with a tobacco clone (NTACS1) (Bailey et al., 1993), and 68% identity with a carnation clone (CARAS1) (Henskens et al., 1994) (Table 2.3). Each had conserved amino acid sequences (Box1~7) showed in ACC synthase (Kende, 1993). The deduced polypeptides encoded by *PPACS1* and *PPACS2* showed 58% identity to each other (Fig. 2.4; Table 2.4). In addition, the deduced amino acids of *PPACS3* revealed 58% and 53% identity to those of *PPACS1* and *PPACS2*, respectively.

#### *Southern blot analysis*

Labeled cDNAs of *PPACS1*, *PPACS2* and *PPACS3* were hybridized to 'Nijisseiki' genomic DNA cut with *Eco*RI or *Hind* III (Fig. 2.5; 2.6; 2.7). Using a *PPACS1* fragment as a probe, digestion of 'Nijisseiki' genomic DNA with *Hind* III (Fig. 2.5, Lane 1) produced three hybridizing genomic fragments at 7.7, 3.5 and 1.0 kb and digestion with *Eco*RI (Fig. 2.5, Lane 2) produced four hybridizing genomic fragments at 12.0, 2.5, 2.0 and 1.5 kb. The *PPACS1* cDNA does contain no *Eco*RI or one *Hind* III site and the PCR-generated genomic fragment (2.2kb) using specific primers (ACS1-1 and ACS1-2) for *PPACS1* amplification contains one *Eco*RI site and one *Hind* III site (Fig. 2.8). Thus, the

Table 2.1 Comparison of amino acid sequences deduced from *PPACS1* and ACC synthase genes of other plant species.

Clone	Plant species	Similarity (%)	Accession No.
pPCACS1	<i>Pyrus communis</i>	98	X87112
MdACS1	<i>Malus domestica</i>	97	U89156
STACS1A	<i>Solanum tuberosum</i>	73	Z27233
STACS1B	<i>Solanum tuberosum</i>	73	Z27234
CMACS2	<i>Cucurbita maxima</i>	73	D01033

Table 2.2 Comparison of amino acid sequences deduced from *PPACS2* and ACC synthase genes of other plant species.

Clone	Plant species	Similarity (%)	Accession No.
MdACS3	<i>Malus domestica</i>	97	U73816
STACS2A	<i>Solanum tuberosum</i>	76	Z27235
DsACS1	<i>Doritaenopsis sp.</i>	61	L07882
CSACS2	<i>Cucumis sativus</i>	61	U59813

Table 2.3 Comparison of amino acid sequences deduced from *PPACS3* and ACC synthase genes of other plant species.

Clone	Plant species	Similarity (%)	Accession No.
GAC2	<i>PelargoniumX hortorum</i>	72	U17231
STACS5	<i>Solanum tuberosum</i>	69	U70842
NTACS1	<i>Nicotiana tabacum</i>	68	X65982
CARAS	<i>Dianthus caryophyllus</i>	68	X66605
STACS4	<i>Solanum tuberosum</i>	67	L20634

Table 2.4 Comparison of amino acid sequences deduced from *PPACS1*, *PPACS2* and *PPACS3*.

Clone	Similarity (%)		
	<i>PPACS1</i>	<i>PPACS2</i>	<i>PPACS3</i>
<i>PPACS1</i>	----	49	58
<i>PPACS2</i>	----	----	53

PPACS1 -----MRMLSRNATFNSHGQDSSYFLGWQEYKPNPYEHVHTNGIIQMGLAENQ  
PPACS2 MAIDIEQQQQSPGLSKIAVSDTHGEDSPYFAGWKAYDENPYHESSNPSGVIQMGLAENQ  
PPACS3 MASSASEN---RLLLSKIATNEKHGENSPYFDGWKNYNQNPFLHTENPEGVIQMGLAENQ  
\*\* : \* : .\*\* : \* .\*\* \*\* : \* : \*\* : \* : . : \*\*\*\*\*

**BOX1**

PPACS1 LCFDLLESWLAKNPEAAAFKKNGE---SIFAEALFQDYHGLPAFKKAMVDFMAEIRGNK  
PPACS2 VSFDLLEKHLSEASNWGSKGSGVSGFRENALFQDYHGLLSFRKAMASFMEQIRGGR  
PPACS3 LSFDLVEEWIRKNPKASICTAEGI---EKFRNVANFQDYHGLPEFRQAIATFMSKARGGR  
. : \*\* : \* : . : \* : \* : : \* : \* : \* : \*\*\*\*\* : \* : \* : . : \*\* : \* : . :

**BOX2**

PPACS1 VTFDPNHLVLTAGATSANETFIIFCLADPGAEFLIPTPYYPGFDRDLKWRGTGVEIVPIHCT  
PPACS2 AKFDPARVVLTAGATAANELLTFIADPGDALLVPTPYYPGFDRDLRWRGTGVNIVPIHCE  
PPACS3 VTFDPHRVMSGGATGANELVMFCLADRGDAFLIPSPYPAFYRDLGWRTGVQIVPVDCC  
. : \*\* : \* : . : \*\* : \* : . : \* : \* : \* : \*\*\*\*\* : \* : \* : \* : \*\*\*\*\* : \* : \*

**BOX3**

PPACS1 NSNGFQITETALEEAYQEAECNLRVKGVLTNPNSPLGTTMTRNELYLLSFVEDKGIH  
PPACS2 SSNNFQITPQALEAAYKEAEAKNMRVRGVLITNPNSPLGATIQRAVLEEILDFVTQKNIH  
PPACS3 SSNNFKITKEALEAAYEKAQNNINVKGLIITNPNSPLGTTLDKDTLESVTFINQKNIH  
. : \*\* : \* : . : \*\* : \* : . : \* : \* : \* : \*\*\*\*\* : \* : \* : \* : \*\*\*\*\* : \* : \*

**BOX4**

PPACS1 LISDEIYSGTAFSSPSFISVMEVLKDRNCNDEFVQQRVHVVSLSKDLGLPGFRVGAIV  
PPACS2 LVSDIYSGSAFSSSEFISVAEILED-----QYKDAERVHIVYSLSKDLGLPGFRVGTIV  
PPACS3 LVCDEIYAATVFSSPKFTCITEVIQNMN-----CNPNIHIVYSLSKDMGFPGLRVGIVY  
\* : . : \*\*\*\*\* : \* : \* : \* : . : \* : \* : \* : \*\*\*\*\* : \* : \* : \* : \*\*\*\*\* : \*

**Box5**

PPACS1 SNDDMVAAATKMSSFGLVSSQTQHLLSAMLSDKKLTKNYIAENHKRLKQRQKNLVSGLQ  
PPACS2 SFNDKVVTARRMSSFTLISSETQHLLASMLSDKEFTGNYIKTNRERLRRRYDMIVEGLK  
PPACS3 SYNDVVNIGRMSSFGLVSSQTQHMLASMLSDDEFVEKFLTTSAKRLAKRHGVFTDGLE  
\* : \* : \* : . : \*\*\*\*\* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \*\*\*\*\* : \*

**BOX6**

PPACS1 KAGISCLNGNAGLFCWVDMRHLLRSNTFEAEMELWKKIVYEVHLNISPGSSSCHCTEPGWF  
PPACS2 KSGIECLKGNAGLFCWMNLSPLDEPTRECELTWDSMLHEVKLNISPGSSSCHCSEPGWF  
PPACS3 EVGINCLKSNAGLFCWMDLRLLKDQTFDGMVLWRVIVNEVKLVNISPSSSFKCPEPGWF  
: \*\* : \* : . : \*\*\*\*\* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \*\*\*\*\* : \*

**BOX7**

PPACS1 RVCFANLPERTLDLAMQRLKAFVGE-YYNVPEVNGRSQSSHLSHRGQSLTKWVSRLSFD  
PPACS2 RVCFANMSEQTLGIALTRIHNFMEK-RERAC-----  
PPACS3 RVCFANMDDTVEVALKRIRAFVGQGKKAQEQVKSPPKRWQSNLRLSFSSSSRRFEQE  
\*\*\*\*\* : \* : \* : \* : \* : \* : \*

PPACS1 DRCPIHGR-----  
PPACS2 -----  
PPACS3 GVSVMSPRMSPHSPMPHSPLLRATT

Fig. 2.4 Comparison of deduced amino acid sequences of *PPACS1*, *PPACS2* and *PPACS3* cDNAs. The seven underlined amino acid residues are conserved regions in ACC synthases from various plant species (Kende, 1993)



number and the size of hybridizing genomic fragments suggest the presence of two or more than two related *PPACS1* genes in Japanese pear. Using a *PPACS2* fragment as a probe, digestion of 'Nijisseiki' genomic DNA with *Hind* III (Fig. 2.6, Lane 1) produced four hybridizing genomic fragments at 7.7, 7.5, 3.0 and 1.2 kb and digestion with *Eco* RI (Fig. 2.6, Lane 2) produced three hybridizing genomic fragments at 5.0, 4.2 and 1.2 kb. The *PPACS2* cDNA does contain two *Eco* RI and one *Hind* III site and the genomic fragment (2.1 kb) PCR-generated using specific primers (ACS2-1 and ACS2-2) for *PPACS2* amplification contains one *Eco* RI site and one *Hind* III site (Fig. 2.9). Thus, the number and size of hybridizing genomic fragments suggest the presence of two or more than two closely related *PPACS2* genes in Japanese pear. Using a *PPACS3* fragment as a probe, digestion of 'Nijisseiki' genomic DNA with *Eco* RI (Fig. 2.7, Lane 1) produced four hybridizing genomic fragments at 6.6, 5.0, 4.5 and 1.9 kb and digestion with *Hind* III (Fig. 2.7, Lane 2) produced six hybridizing genomic fragments at 10, 6.6, 4.5, 3.5, 2.7 and 2.5 kb. The cDNA does not contain an *Eco* RI or a *Hind* III site and the PCR-generated genomic fragment (2.7kb) using specific primers (ACS3-1 and ACS3-2) for *PPACS3* amplification contains no *Eco* RI site and one *Hind* III site (Fig. 2.10). Thus, the number and the size of hybridizing genomic fragments suggest the presence of two or more than two closely related *PPACS3* genes in Japanese pear.

#### *Expression analysis of ACC synthase genes*

Total RNA from ripe fruits of various Japanese pear cultivars was

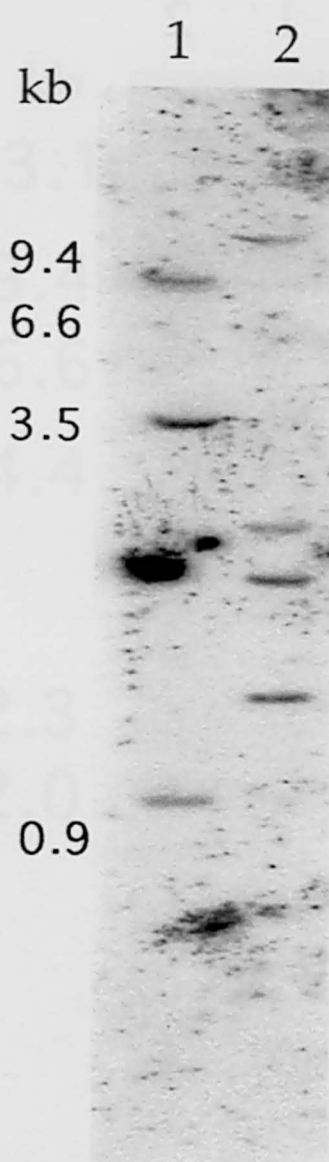


Fig. 2.5 Southern analysis, using probe PPACS1, of *Hind*III-(Lane 1) and *Eco*RI-(Lane 2) digested DNAs from 'Nijisseiki'

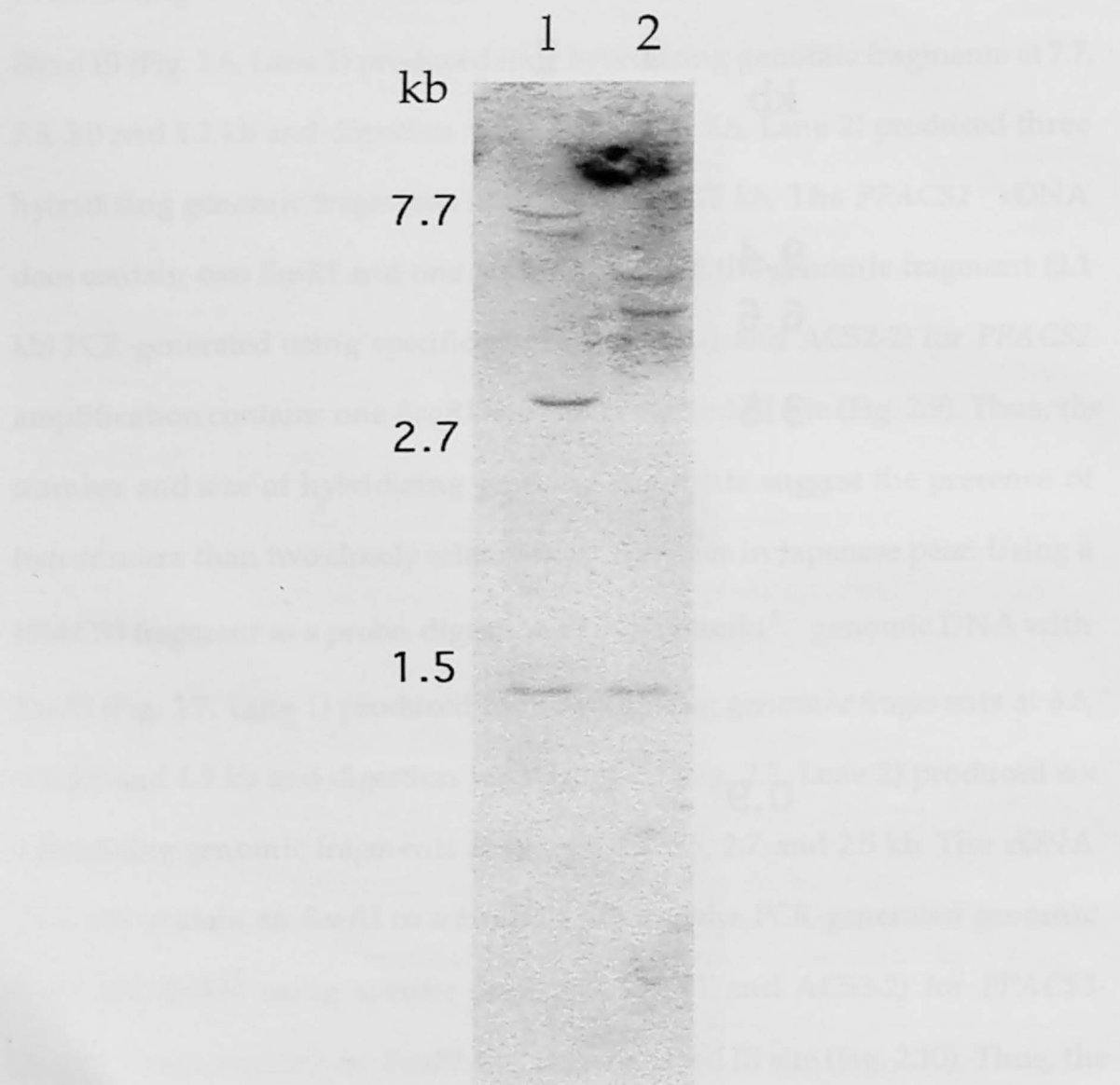


Fig. 2.6 Southern analysis, using probe PPACS2, of *Hind*III-(Lane 1) and *Eco*RI-(Lane 2) digested DNAs from 'Nijisseiki'

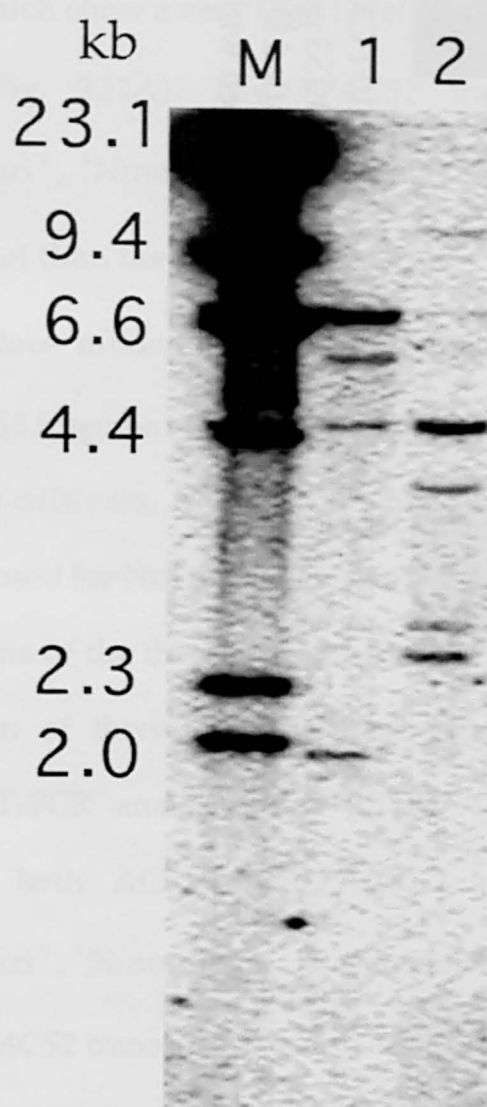


Fig. 2.7 Southern analysis, using probe PPACS3, of *Eco*RI-(Lane 1) and *Hind*III-(Lane 2) digested DNAs from 'Nijisseiki'

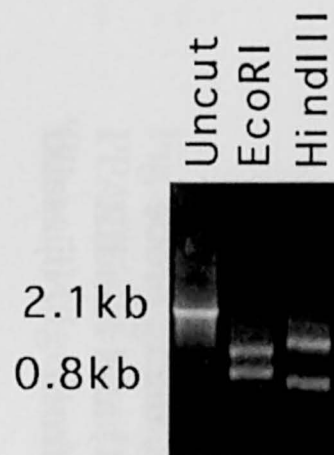


Fig. 2.8 PCR-RFLP analysis of *PPACS1* gene in 'Nijisseiki'. Amplified DNA fragments were digested with *EcoRI* and *HindIII*.

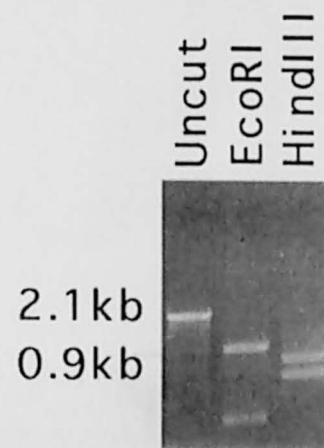


Fig. 2.9 PCR-RFLP analysis of *PPACS2* gene in 'Nijisseiki'. Amplified DNA fragments were digested with *EcoRI* and *HindIII*.

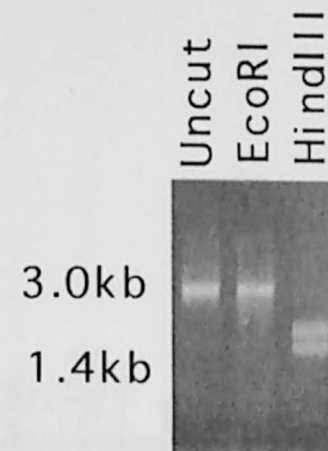


Fig. 2.10 PCR-RFLP analysis of *PPACS3* gene in 'Nijisseiki'. Amplified DNA fragments were digested with *EcoRI* and *HindIII*.

prepared and examined by Northern blot analysis to analyze the expression of three ACC synthase genes, (Fig. 2.11A). *PPACS1* was expressed in ripening fruits of 'Ninomiyahakuri', 'Ninomiya', 'Rokugatsu', 'Edoya' and 'Awayuki', which show a very high level of ethylene production ( $\geq 10 \mu$  l/kg f.w./hr) (Fig. 2.11A). The *PPACS2* transcript was detected in 'Ninomiyahakuri', 'Ninomiya', 'Kikusui', 'Chojuro' and 'Shinsui', but at a lower level than the *PPACS1* transcript. 'Kikusui', 'Chojuro' and 'Shinsui' produce moderate levels of ethylene during fruit ripening. While the *PPACS3* fragment was used as a probe, no signals were detected in ripe fruits of any cultivars. An RT-PCR experiment using cDNA templates from total RNA used for Northern analysis was performed to re-examine the expression patterns of the three ACC synthase genes (Fig. 2.11B). Differences in the expression of three ACC synthase genes with the cultivar were examined by RT-PCR analysis (Fig. 2.11B). The *PPACS1* transcript was amplified with both ACS1-1 and ACS1-2 primers from cDNAs of 'Ninomiyahakuri', 'Ninomiya', 'Rokugatsu', 'Edoya' and 'Awayuki' fruits and the *PPACS2* transcript was also amplified with ACS2-1 and ACS2-2 primers from cDNAs of 'Ninomiyahakuri', 'Ninomiya', 'Kikusui', 'Chojuro' and 'Shinsui' fruits. These results are in accordance with the Northern blot analysis. The *PPACS3* transcript was detected in ripe fruits of all the cultivars using specific primers ACS3-1 and ACS3-2. These data show that the *PPACS3* mRNA is expressed at an extremely low level in ripening

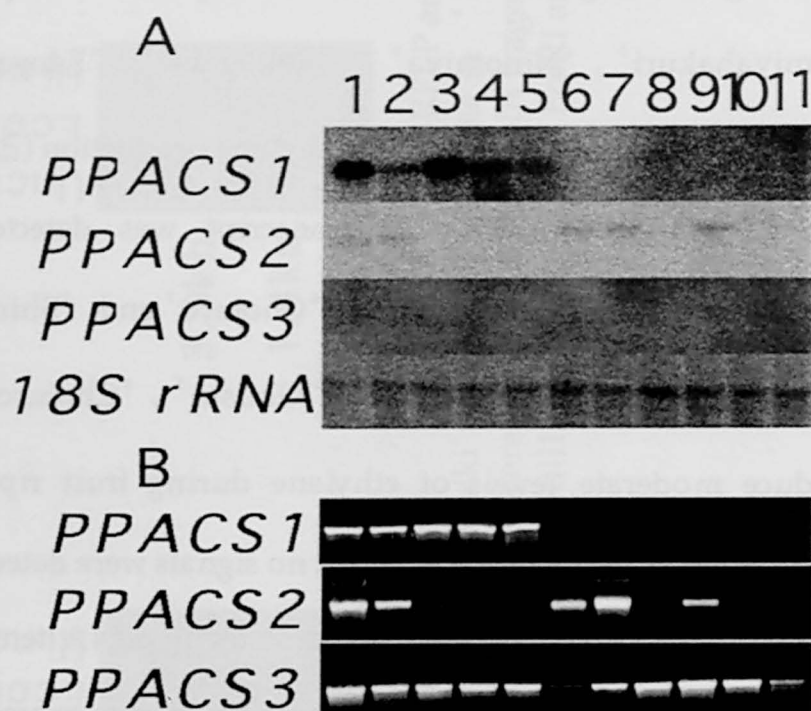


Fig.2.11 Expression of three ACC synthase genes during fruit ripening of 11 cultivars showing various levels of ethylene. Lane 1: 'Ninomiya', 2: 'Ninomiyahakuri', 3: 'Edoya', 4: 'Rokugatsu', 5: 'Awayuki', 6: 'Shinsui', 7: 'Kikusui', 8: 'Nijisseiki', 9: 'Chojuro', 10: 'Shinsetsu', 11: 'Hosui'. 'Ninomiya', 'Ninomiyahakuri', 'Edoya', 'Rokugatsu' and 'Awayuki' are high ethylene producers (more than  $10 \mu\text{l C}_2\text{H}_4/\text{kg/hr}$ ); 'Shinsui', 'Kikusui' and 'Chojuro' are moderate ethylene producers ( $0.5 \mu\text{l C}_2\text{H}_4/\text{kg/hr} \sim 10 \mu\text{l C}_2\text{H}_4/\text{kg/hr}$ ); 'Nijisseiki', 'Shinsetsu' and 'Hosui' are low ethylene producers (less than  $0.5 \mu\text{l C}_2\text{H}_4/\text{kg/hr}$ ). (A) Northern blot analysis of total RNA ( $10 \mu\text{g}$ ). An 18S rDNA probe was used to estimate RNA loading. (B) RT-PCR analysis using three sets of specific primers.

fruits of all cultivars of Japanese pear. Moreover, the expression of the three ACC synthase genes in response to wounding was monitored by Northern blot analysis. Wounding treatments resulted in the accumulation of *PPACS3* but not of *PPACS1* and *PPACS2* mRNA (Fig. 2.12). The *PPACS3* mRNA starts to accumulate 6 hr after wounding and continues to maintain a high level for 24 hr. These results indicate that the *PPACS3* gene is induced by wounding.

## Discussion

cDNAs from Japanese pear fruit which encode for ACC synthase and are ripening-related were isolated. The identity as ACC synthase was confirmed by the seven highly conserved domains found in ACC synthase of other plant species (Kende, 1993). As reported for tomato (Oetiker et al., 1997), potato (Destefano-Beltran et al., 1995), mung bean (Kim et al., 1997), and other species, the ACC synthase is encoded by a multigene family. This is also the case for Japanese pear in which three ripening-related ACC synthase cDNA clones were isolated. Two of them, *PPACS1* and *PPACS2*, share 98% and 97% sequence identities with apple ACC synthase cDNA clones, *Md-ACS1* and *Md-ACS3*, respectively (Lay-Yee, et al., 1995; Rosenfield et al., 1996). In apple, to date, three ACC synthase cDNAs (*Md-ACS1*, *Md-ACS2* and *Md-ACS3*) have been cloned. None of the three ACC synthases in apple shares a significant sequence identity with *PPACS3*. Also, there is a high degree of conservation of most coding regions between pear and apple. For example, the coding regions of a pear ACC oxidase cDNA (pPC-ACO-1), a pear



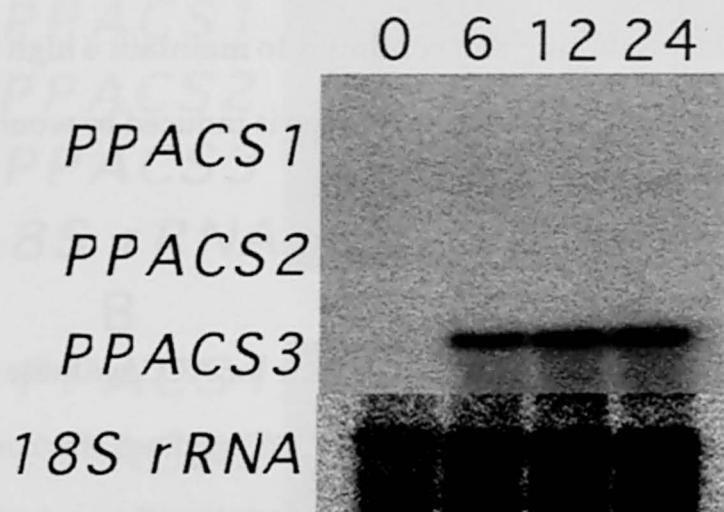


Fig. 2.12 Northern blot analysis of three ACC synthase genes 24h after wounding in overripening fruit of 'Nijisseiki'. 0: control, 6: 6h after wounding, 12: 12h after wounding, 24: 24h after wounding. An 18S rDNA probe was used to estimate RNA loading.

polygalacturonase inhibitor cDNA and a pear polyphenol oxidase cDNA are 98%, 97% and 93% identical to those of apple homologous counterpart clones (Dong et al., 1992; Haruta et al., 1999; Lelievre et al., 1997b; Ross et al., 1992; Stotz et al., 1993). Thus, for any one of the pear genomic or cDNA sequences tested, a homologous counterpart is expected in apple. Based on the evidence that a *PPACS3* homologous gene lacks in apple gene family, it is supposed that *Md-ACS4* exist as an additional isoform in the apple genome.

The expression of the three ACC synthase genes in the ripe fruit of several cultivars was examined at the peak of ethylene production to elucidate the cause of the differences in ethylene production. RNA was isolated from ripening fruits of cultivars producing high levels of ethylene ( 'Ninomiyahakuri' , 'Ninomiya' , 'Rokugatsu' , 'Edoya' and 'Awayuki' ), moderate levels of ethylene ( 'Shinsui' , 'Kikusui' and 'Chojuro' ), and low levels of ethylene ( 'Nijisseiki' , 'Shinsetsu' and 'Hosui' ). *PPACS1*, *PPACS2* and *PPACS3* fragments were used as probes. The 2.0 kb transcripts of *PPACS1* were highly expressed in the ripening fruit of 'Ninomiyahakuri' , 'Ninomiya' , 'Rokugatsu' , 'Edoya' , and 'Awayuki' , but not in 'Shinsui' , 'Kikusui' , 'Chojuro' , 'Nijisseiki' , 'Shinsetsu' and 'Hosui' fruits. The 2.0 kb transcripts of *PPACS2* were detected in 'Ninomiyahakuri' , 'Ninomiya' , 'Shinsui' , 'Kikusui' and 'Chojuro' fruits, but to a lesser extent than *PPACS1*. *PPACS2* transcripts were not detected in 'Edoya' , 'Rokugatsu' , 'Awayuki' ,

'Nijisseiki', 'Shinsetsu' and 'Hosui' fruit. 'Ninomiyahakuri' and 'Ninomiya' fruit were the cultivars to express both *PPACS1* and *PPACS2*. This indicates that the expression of *PPACS1* and *PPACS2* is differentially regulated in Japanese pear cultivars. The expression of *PPACS3* in ripening fruit was not detected by Northern blot analysis, but was detected by RT-PCR analysis. This indicates that *PPACS3* was expressed at a very low level in Japanese pear. The *PPACS3* transcripts were expressed in all cultivars during fruit ripening regardless of cultivar differences in the amount of ethylene synthesis. The expression of *PPACS1* was specific to cultivars that showed a very high level of ethylene production and that of *PPACS2* was specific to 'Ninomiyahakuri' and 'Ninomiya' and to cultivars which showed a moderate level of ethylene production.

Wounding of ripening fruit resulted in the accumulation of *PPACS3*, but not of *PPACS1* and *PPACS2* transcripts. RNA isolated from wounded fruit contained ACC synthase mRNA detectable by hybridization with *PPACS3*. Nakatsuka et al. (1998) reported four genes (*LE-ACS1A*, *LE-ACS2*, *LE-ACS3* and *LE-ACS4*) for ACC synthase were expressed in tomato during fruit ripening. Two of them, *LE-ACS2* and *LE-ACS4* showed ripening-specific expression. Both of these genes responded to wound signals (Olson et al., 1991). This suggests that *PPACS3* functions as *LE-ACS2* and *LE-ACS4* gene in tomato.

Cultivars showing a high level of ethylene production ( $\geq 10 \mu\text{l/kg f.w./hr}$ ) during fruit ripening expressed either two ACC synthase genes

(*PPACS1* and *PPACS3*) or three genes (*PPACS1*, *PPACS2* and *PPACS3*). Those showing a moderate level ( $0.5 \mu\text{l/kg f.w./hr} \sim 10 \mu\text{l/kg f.w./hr}$ ) expressed both *PPACS2* and *PPACS3* genes and those showing a low level ( $\leq 0.5 \mu\text{l/kg f.w./hr}$ ) expressed a *PPACS3* gene only.

## **2.2 Isolation of a cDNA encoding for 1-aminocyclopropane-1-carboxylate oxidase from ripening fruits and differences in the gene expression during fruit ripening with the cultivar**

### **Introduction**

The second step in the ethylene biosynthetic pathway is catalyzed by ACC oxidase. cDNAs coding for ACC oxidase have been cloned and sequenced from a number of species including apple (Dong et al., 1992; Ross et al., 1992), melon (Balague et al., 1993), tomato (Barry et al., 1996; Hamilton et al., 1991; Nakatsuka et al., 1998; Spanu et al., 1991), avocado (McGarvey et al., 1990) and kiwifruit (Whittaker et al., 1997). These studies have shown that ACC oxidase is encoded by a multigene family whose various members are differentially regulated by many factors.

In the ethylene biosynthetic pathway, ACC synthase is generally regarded as the rate-limiting step (Yip et al., 1992). However, Whittaker et al. (1997) reported that ACC oxidase activity was limiting to ethylene biosynthesis in kiwifruit in the climacteric. Furthermore, the ability of extending shelf life by inhibiting ethylene biosynthesis using an antisense ACC oxidase gene has been demonstrated in tomato (Hamilton et al., 1990). In order to extend the storage life of Japanese pear fruit by inhibiting ethylene synthesis, molecular studies on ACC oxidase in Japanese pear are needed. However, little is known about molecular control and characterization of Japanese pear ACC oxidase.

Here, I report the isolation of a Japanese pear ACC oxidase cDNA clone as the first step in molecular analysis of this enzyme in Japanese pear.

## Materials and methods

### *cDNA library construction*

Japanese pear fruit cv. Chojuro were harvested from the orchard of Tottori University (about 130 days after anthesis). The harvested fruit was transferred to the room kept at 20°C immediately. Total RNA and poly(A)<sup>+</sup>RNA were prepared from 'Chojuro' fruit 4 days after harvest. cDNA library was constructed from 5 $\mu$ g poly(A)<sup>+</sup>RNA using the Time Saver cDNA synthesis kit (Pharmacia). cDNA fragments were inserted into the *Eco*RI and *Not*I site of the  $\lambda$ Excell (Pharmacia). Phages were packaged and plated on *E. coli*. NM522 according to the manufacturer's instruction. Approximately total 4 $\times$ 10<sup>4</sup> plaques were screened.

### *Probe preparation and library screening*

A hybridization probe for screening a cDNA library was prepared by RT-PCR strategy. The 31-mer oligodeoxy-nucleotide (I) 5'-CCCCGCATGC(CG)A(AG)AA(CT)TGGGG(CT)TT(CG)T(AT)(CT)GAG3' and the 29-mer oligodeoxy-nucleotide (II) 5'-GGGGGTCGACTC(AG)AA(CGT)C(GT)(CT)GG(CT)TC(CT)TT(AGCT)G3',

corresponding to the highly conserved regions (80~200 bp and 880~1010 bp, respectively) of ACC oxidase from 8 plant species, were used as primers for DNA amplification. One microgram poly(A)<sup>+</sup>RNA was converted to first-strand cDNA using a T-primed First-strand Kit (Pharmacia). Using the constructed first-strand cDNA as a template, oligodeoxynucleotide I as an upstream primer, and oligodeoxynucleotide II as a downstream primer, approximately 800 bp DNA products were amplified by PCR (40 cycles denaturing, annealing, and polymerization were carried out at 94°C for 1min, 45°C for 1 min, and 72°C for 2 min, respectively). PCR products were purified from a 0.7% agarose gel and used as a probe after <sup>32</sup>P-labeling with a random prime labeling system. The filters with transferred plaques were hybridized with the <sup>32</sup>P-labeled probe in 40% formamide, 5X SSPE, 5X Denhardt's solution, 0.1% SDS, 0.1% blocking reagent (Boehringer Mannheim), and 200  $\mu$ gml<sup>-1</sup> salmon sperm DNA at 42°C overnight and then washed three times with 0.2 X SSC and 0.1% SDS at 65°C for 15 min.

#### *Cloning of an ACC oxidase cDNA and DNA sequence analysis*

Using the PCR-generated fragment as a probe on the cDNA library, ca. 2.5% of the total clones gave positive signals. Ten of these were selected and the longest cDNA insert, *PPAOX1*, was sequenced.

A selected positive plaque was subcloned into pBluescript II KS+ plasmid vector (Stratagene) and the cDNA insert was sequenced by the dideoxy chain termination methods using a Bca Best Sequencing Kit (Takara Shuzo) with

an automatic DNA sequencer (ALF II DNA Sequencer, Pharmacia). Progress deletions were generated by exonuclease digestions using a nested deletion kit (Pharmacia). The nucleotide sequence was processed by a DNASIS software package (Hitachi). The nucleotide sequence reported in this chapter is available from the DDBJ data library under the accession number D67038.

#### *Northern blot analysis*

Total RNA was prepared from 'Chojuro' fruit of 0,4,9,12 days after harvest. To analyze the cultivar differences in gene expression, the fruits of 8 cultivars ('Ninomiya', 'Edoya', 'Awayuki', 'Kikusui', 'Nijisseiki', 'Chojuro', 'Shinsetsu' and 'Hosui') were harvested at the ripening stages. For gene expression analysis, total RNA was extracted from these samples as described before. Ten  $\mu$ g of total RNA was fractionated on formaldehyde agarose gel and transferred onto a Hybond N+ nylon membrane filter (Amersham). The filter with transferred RNA was hybridized with the  $^{32}$ P-labeled probe in 40% formamide, 5 X SSPE, 5 X Denhardt's solution, 0.1% SDS, 0.1% blocking reagent, and 200  $\mu$ gml<sup>-1</sup> salmon sperm DNA at 42°C overnight. After being washed twice for 15 min with 2 x SSC containing 0.1% SDS and three times with 0.2 X SSC and 0.1% SDS at 65°C for 15 min, the membrane was exposed to X-ray film.

#### *Southern blot analysis and PCR-RFLP analysis*



Total DNA was extracted from fresh leaves of 'Chojuro' according to the method of Teramoto et al. (1994). Ten  $\mu$ g of DNA was digested with *Eco*RI and *Dra*I and separated on 0.7% agarose gels, then transferred to a nylon membrane as described above. The membrane was hybridized overnight at 42°C in 40% formamide condition, and then washed three times with 0.2 X SSC and 0.1% SDS at 65°C for 15 min following twice with 2 X SSC and 0.1% SDS at 65°C for 15 min. For PCR analysis, based on sequencing data of *PPAOX1*, forward (AOX1-1: 5'-TACACACAGAGCATAAAAAACCAAG-3') and reverse (AOX1-2: 5'-TGTGATATTACTAATCTGTATAAC-3') primers were designed to amplify genomic DNA fragments from 'Chojuro'. The amplification was carried out for 40 cycles of 60s at 94°C, 60s at 50°C and 90s at 72°C, with final cycle at 72°C for 10min. Amplified fragments were digested with *Eco*RI and *Dra*I, separated on 0.7% agarose gels and stained with ethidium bromide.

## Results and discussion

The cDNA insert (*PPAOX1*) was 1288 bp in length, and contained an open reading frame of 942 bp (Fig. 2.13). This open reading frame encoded a protein of 314 amino acids. The predicted molecular mass of the protein encoded by *PPAOX1* is 35kD. The predicted polypeptide showed 97% amino acid identity with an apple clone (pAP4,pAE12) (Dong et al., 1992; Ross et al., 1992), 85% identity with a tomato clone (pTOM13) (Hamilton et al., 1990), 81% identity

1	TACACACAGAGCATAAAAACCAAGCAATCCAAAGAGAA	39
40	ATGGCGACCTTCCCAGTTGTTGACTTGAGCCTTGTCATGGTGAAGAGAGAGTAGCAACC	99
1	M A T F P V V D L S L V N G E E R V A T	20
100	TTGGAGAAGATCAATGATGCTTGTGAGAACTGGGGTTTCTTTGAGTTGGTGAACCATGGG	159
21	L E K I N D A C E N W G F F E L V N H G	40
160	ATATCTACTGAGCTTTTGGACACTGTGGAGAAGATGACCAAGGATCACTACAAGAAGACC	219
41	I S T E L L D T V E K M T K D H Y K K T	60
220	ATGGAGCAAAGGTTTAAGGAAATGGTGGCAGCCAAAGGCCTCGACGCTGCCAGTCCGAA	279
61	M E Q R F K E M V A A K G L D A V Q S E	80
280	ATCCACGACTTGGAGTGGGAAAGCACCTTCTTCTTGCGCCACCTTCTTCTCAACATT	339
81	I H D L D W E S T F F L R H L P S S N I	100
340	TCCGAAGTCCCTGATCTCGAGGAAGATTACAGGAAGACCATGAAAGAATTGCACTGGAA	399
101	S E V P D L E E D Y R K T M K E F A V E	120
400	CTGGAGAAGCTAGCTGAGAAGCTTTTGGACTTGCTGTGTGAGAATCTTGGACTCGAGAAG	459
121	L E K L A E K L L D L L C E N L G L E K	140
460	GGTTATCTGAAGAAGGTTTCTATGGATCCAAGGGTCCGAATTTGGGACCAAGGTCAGC	519
141	G Y L K K V F Y G S K G P N F G T K V S	160
520	AACTACCTCCATGCCCAAGCCAGACCTGATCAAGGGACTCCGGGCCACAGCGACGCC	579
161	N Y P P C P K P D L I K G L R A H S D A	180
580	GGTGGCATCATCTGCTTTTCCAGGATGACAAGGTCAGCGGCCTCCAGCTTCTCAAGGAT	639
181	G G I I L L F Q D D K V S G L Q L L K D	200
640	GGTGAATGGGTGGATGTCCCCCAATGCACCACTCCATTGTCATAAACTTAGGTGACCAG	699
201	G E W V D V P P M H H S I V I N L G D Q	220
700	ATTGAGGTGATACCAATGGGAAGTACAAGAGTGTGATGCACCGGGTGATAGCTCAGTCG	759
221	I E V I T N G K Y K S V M H R V I A Q S	240
760	GACGGGACCAGAATGTCGATAGCCTCGTTCTACAACCCAGGCGATGATGCCTTTATCAGC	819
241	D G T R M S I A S F Y N P G D D A F I S	260
820	CCAGCACCGGCAGTGCTTGAGAAGAAAAGTGGGACGCCCACTTATCCCAAGTTTGTG	879
261	P A P A V L E K K T E D A P T Y P K F V	280
880	TTTGATGACTACATGAAGCTGTATTCTGGCCTGAAATCCAAGCCAAGGAACCAAGATT	939
281	F D D Y M K L Y S G L K F Q A K E P R F	300
940	GAAGCTATGAAGGCCAAGGAATCGACCCCTGTTGCAACTACCTGAGCTCTGAGAACCGTC	999
301	E A M K A K E S T P V A T T *	
1000	CGATGGAACATGACACAAATATAATAAAAAATTAGTGCTTGGGTTTTATGCGTTTGTGCC	1059
1060	AAATAAAGTTGGGGAGAAGAAAGTTGGGTTTATTTGTTTTCTTTATTTTTTACCTCC	1119
1120	ACTGTTTTTGTGGTTGAAATCTATCTATATAAAATACAAATGATTAGATCCAAATACAA	1179
1180	ATAATTAGATGCCGAAAAGTGGAGGACCCATCACCATGTTCTTCAAAATTTTCATTACG	1239
1240	AAAATATGTTATACAGATTAGTAATATCACAAAAA	

Fig. 2.13 Nucleotide and deduced amino acid sequences of *PPAOX1*.

with a avocado clone (McGarvey et al., 1990), and 74% identity with a melon clone (pMEL1) (Balague et al., 1993). The high sequence homology suggests that the cDNA clone (*PPAOX1*) encodes ACC oxidase in Japanese pear fruit. Especially, the nucleotide and the deduced amino acid sequences are very highly homologous with those of apple ACC oxidase cDNA. Japanese pear and apple belong to the family of Rosaceae. This finding indicates that ACC oxidase is highly conserved in the family of Rosaceae.

cDNAs coding ACC oxidase in tomato (pTOM13) and apple (pAP4) are members of a multigene family (Holdsworth et al., 1988). Using a *PPAOX1* fragment as a probe, digestion of 'Chojuro' genomic DNA with *DraI* (Fig. 2.14, Lane 1) produced two hybridizing genomic fragments at 6.0 and 2.1 kb and digestion with *EcoRI* (Fig. 2.14, Lane 2) produced three hybridizing genomic fragments at 7.7, 3.5 and 3.3kb. The *PPAOX1* cDNA has neither an *EcoRI* nor *DraI* site and the PCR-generated genomic fragment (1.9kb) using specific primers (AOX1-1 and AOX1-2) for *PPAOX1* amplification contains no *EcoRI* and *DraI* sites (Fig. 2.15). The number and the size of hybridizing genomic fragments suggest the presence of multiple copies of closely related *PPAOX1* genes in Japanese pear.

Using Northern analysis, a 1.3kb mRNA homologous to *PPAOX1* was not detected in fruit RNA taken on the day of harvest (Fig.2.16). However, strong signals were detected in fruit 4,9,12 days after harvest. As ethylene is synthesized in these situations, the expression of *PPAOX1* transcripts is positively correlated with the ethylene level. This showed that the transcript

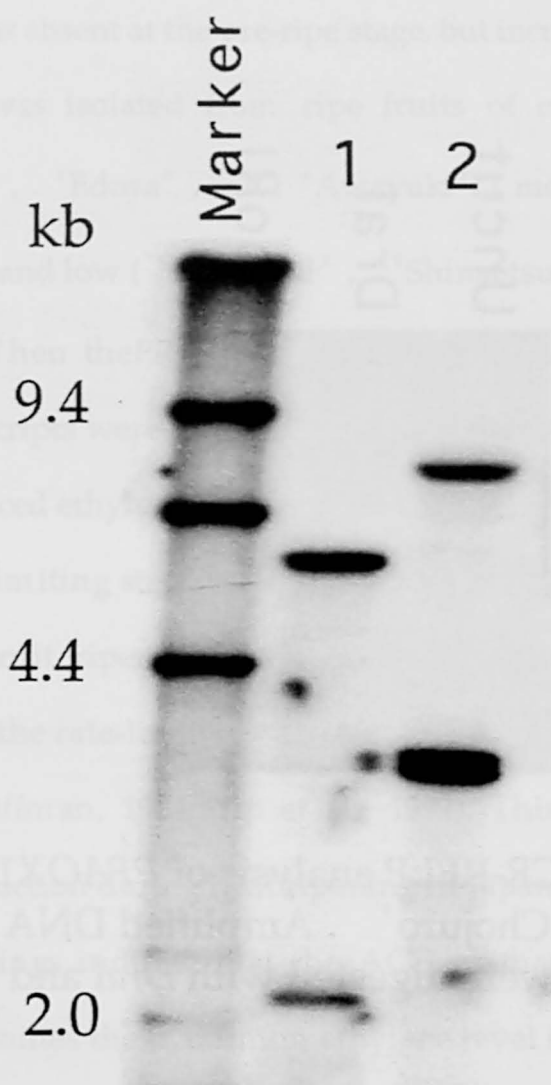


Fig. 2.14 Southern analysis, using probe PPAOX1, of *Dra*I-(Lane 1) and *Eco*RI-(Lane 2) digested DNAs from 'Chojuro'

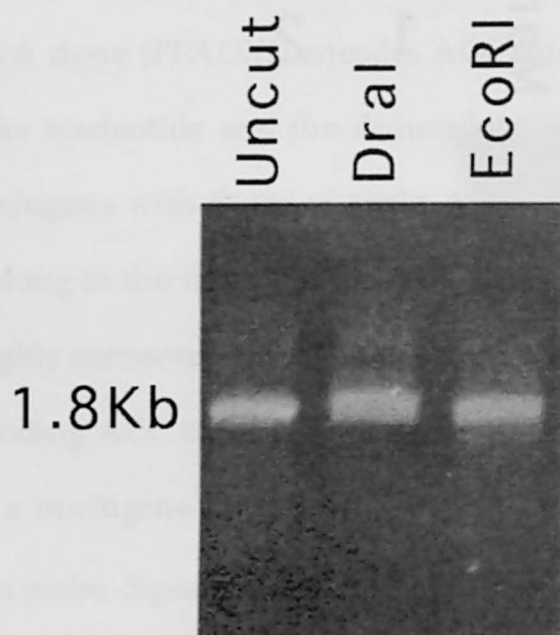


Fig. 2.15 PCR-RFLP analysis of *PPAOX1* gene in 'Chojuro'. Amplified DNA fragments were digested with *DraI* and *EcoRI*.

of this gene was absent at the pre-ripe stage, but increased dramatically in ripe fruits. RNA was isolated from ripe fruits of cultivars producing high ( 'Ninomiya' , 'Edoya' , and 'Awayuki' ), moderate ( 'Kikusui' and 'Chojuro' ), and low ( 'Nijisseiki' , 'Shinsetsu' and 'Hosui' ) levels of ethylene. When the *PPAOX1* cDNA fragment was used as a probe, high *PPAOX1* transcripts were detected in all cultivars, regardless of whether the cultivar produced ethylene or not (Fig.2.17). This shows that the ACC oxidase gene is not a limiting step in the ethylene biosynthetic pathway of Japanese pear during fruit ripening. In plant tissues, ACC synthase is generally regarded to be the rate-limiting enzyme in the ethylene biosynthetic pathway (Yang and Hoffman, 1984; Yip et al., 1992). This concept is applicable to ethylene production during fruit ripening in Japanese pear.

These findings indicate that the ACC synthase reaction, not the ACC oxidase, determines the maximum ethylene level in Japanese pear cultivars.

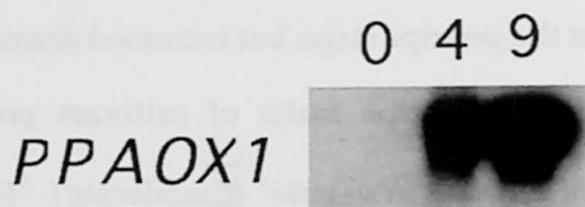


Fig. 2.16 PPAOX1 mRNA levels during fruit ripening in 'Chojuro'. Numbers indicate the days after commercial harvest.

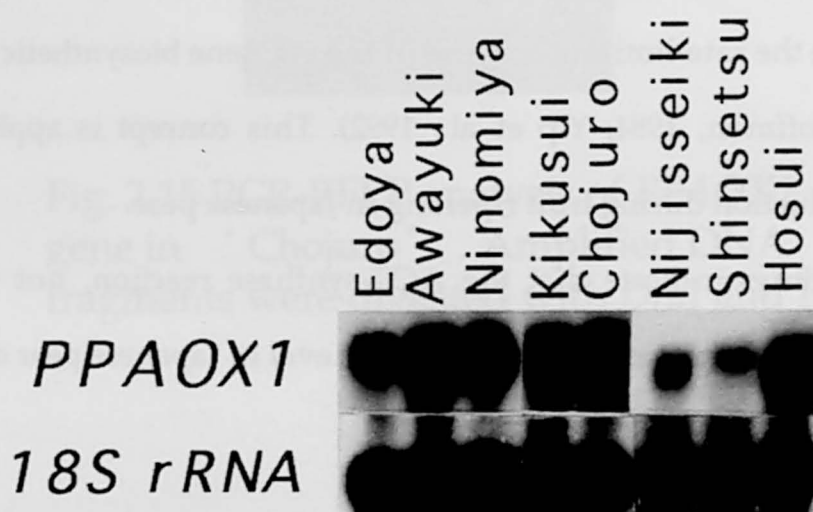


Fig.2.17 Northern analysis using probe PPAOX1, of total RNAs ( $10\mu\text{g}$ ) from ripening fruit of 8 cultivars showing various levels of ethylene during fruit ripening. 'Edoya', 'Awayuki', and 'Ninomiya' have high levels (more than  $10\mu\text{l C}_2\text{H}_4/\text{kg/hr}$ ); 'Chojuro' and 'Kikusui' have moderate levels ( $0.5\mu\text{l C}_2\text{H}_4/\text{kg/hr} \sim 10\mu\text{l C}_2\text{H}_4/\text{kg/hr}$ ); 'Nijisseiki', 'Shinsetsu' and 'Hosui' have low levels (less than  $0.5\mu\text{l C}_2\text{H}_4/\text{kg/hr}$ ). An 18S rDNA probe was used to estimate RNA loadings.

## Chapter 3.

### The mode of inheritance and development of the gene diagnosis of ethylene production during fruit ripening

#### 3.1 RFLP analysis of Japanese pear cultivars using genes encoding for ethylene biosynthetic enzymes as probes

##### Introduction

In the previous chapter, three ACC synthase cDNAs (*PPACS1*, *PPACS2*, *PPACS3*) and a ACC oxidase cDNA (*PPAOX1*) were isolated. Furthermore, it is shown that the *PPACS1* is specially expressed in the cultivars with a high ethylene production level and that the *PPACS2* is specially expressed in the cultivars with moderate ethylene production level during fruit ripening. While, the *PPACS3* transcript is expressed in all cultivars during fruit ripening regardless of the cultivar differences in the amount of ethylene synthesis..

In this chapter, two alleles closely related to the high ethylene levels in Japanese pear fruit during ripening were identified using three ACC synthase cDNAs and a ACC oxidase cDNA isolated, as described in the previous chapter.



## Materials and methods

### *Plant materials*

Thirty-five cultivars of Japanese pear grown at the orchard of Tottori University were used (Table 3.1).

### *DNA isolation and Southern blot analysis*

Total DNA was extracted from the immature leaves of 35 cultivars as described above by the modified SDS method (Teramoto et al., 1994). Ten  $\mu$ g of DNA from each cultivar was digested with *Eco*RI, *Hind* III, *Eco*RV and *Bam*HI, separated by electrophoresis on 0.9% agarose gels, and transferred to Hybond N<sup>+</sup> nylon membranes by Southern blotting. DNA was fixed by UV crosslinking. Membranes were hybridized with <sup>32</sup>P-labeled inserts from *PPAOX1*, *PPACS1*, *PPACS2* and *PPACS3* at 42°C overnight in buffer containing 40% formamide, 5 x SSPE, 5 x Denhardt's, and 0.1% SDS. Post hybridization washes were 2 x 15 minutes at 68°C in 0.2 x SSC and 0.1% SDS, after which the membranes were exposed to X-ray film.

## Results

### *Southern blot analysis using PPACS1 as a probe*

RFLP analysis of Japanese cultivars with *PPACS1* as a probe against *Eco*RV and *Bam*HI digests showed no length polymorphisms. When DNA was digested with *Hind* III, all cultivars showed hybridizing bands of 7.7, 3.5, and

**Table 3.1 Fruit ethylene production and corresponding marker types in 35 Japanese pear cultivars**

Cultivars (Maturing time)	PPACS1 <sup>a</sup> (2.8kb)	PPACS2 <sup>b</sup> (0.8kb)	RFLP type <sup>c</sup>	Maximum Ethylene level <sup>d</sup>
Ninomiya (Early)	+	+	AB	High
Edoya (Early)	+	-	Ab	High
Okuroku (Early)	+	-	Ab	High
Rokugatsu (Early)	+	-	Ab	High
Awayuki (Mid)	+	-	Ab	High
Sotoorihime (Mid)	+	-	Ab	High
Akaho (Early)	-	+	aB	Moderate
Ishiiwase (Early)	-	+	aB	Moderate
Kimitsukawase (Early)	-	+	aB	Moderate
Kosui (Early)	-	+	aB	Moderate
Shinchu (Early)	-	+	aB	Moderate
Shinsui (Early)	-	+	aB	Moderate
Yakumo (Early)	-	+	aB	Moderate
Chojuro (Mid)	-	+	aB	Moderate
Doitsu (Mid)	-	+	aB	Moderate
Gion (Mid)	-	+	aB	Moderate
Kikusui (Mid)	-	+	aB	Moderate
Kindhaku (Mid)	-	+	aB	Moderate
Kozo (Mid)	-	+	aB	Moderate
Suisei (Mid)	-	+	aB	Moderate
Wasekoso (Mid)	-	+	aB	Moderate
Inugoroshi (Late)	-	+	aB	Moderate
Ohiromaru (Late)	-	+	aB	Moderate
Hosui (Mid)	-	-	ab	Low
Nijisseiki (Mid)	-	-	ab	Low
Sekaiichi (Mid)	-	-	ab	Low
Amanokawa (Late)	-	-	ab	Low
Imamuraaki (Late)	-	-	ab	Low
Konpeito (Late)	-	-	ab	Low
Niitaka (Late)	-	-	ab	Low
Okusankichi (Late)	-	-	ab	Low
Ruisannashi (Late)	-	-	ab	Low
Shinsetsu (Late)	-	-	ab	Low
Waseaka (Late)	-	-	ab	Low
Yokogoshi (Late)	-	-	ab	Low

a: +, the presence of 2.8kb band, -, the absence of 2.8kb band

b: +, the presence of 0.8kb band, -, the absence of 0.8kb band

c: A, linked to high level of ethylene and B, linked to moderate level of ethylene

d: Maximum ethylene production during fruit ripening

High: >10  $\mu$ L/kg/hr

Moderate: 0.5  $\mu$ L/kg/hr ~ 10  $\mu$ L/kg/hr

Low: <0.5  $\mu$ L/kg/hr

1.0kb (Fig. 3.1) and cultivars 'Ninomiya', 'Rokugatsu', 'Edoya', 'Okuroku', 'Awayuki', and 'Sotoorihime' showed an additional band of 2.8kb, indicating an extra copy of *PPACS1*. Cultivars showing the additional 2.8kb band also produced very high levels of ethylene (more than 10  $\mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr.}$ ) during fruit ripening. The extra copy therefore distinguishes high ethylene producers from moderate and low level ethylene producers. Southern blot analysis indicates that in these cultivars, the additional *PPACS1* gene resulted from a gene duplication event similar to those shown in potato (Destefano-Beltran et al., 1995) and cucumber (Trebitsh et al., 1997). *Eco*RI digests of genomic DNA from the same high ethylene producing cultivars yielded an extra band of 9.4kb for 'Edoya' and 'Rokugatu' and a 6.0kb band for 'Ninomiya', 'Okuroku', 'Rokugatu', 'Sotoorihime' and 'Awayuki' (Fig. 3.2). This finding also supports the gene duplication hypothesis mentioned above, and suggests that the additional ACC synthase gene, homologous to *PPACS1*, operates to produce high levels of ethylene during fruit ripening.

#### *Southern blot analysis using PPACS2 as a probe*

Southern analysis with *PPACS2* revealed a RFLP within some Japanese pear cultivars (Fig. 3.3). Cultivars of moderate ethylene production (0.5~10  $\mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr.}$ ; 'Shinsui', 'Kosui', 'Chojuro', 'Kikusui', 'Yakumo' and further 13 cultivars) contained an extra-hybridizing *Hind*

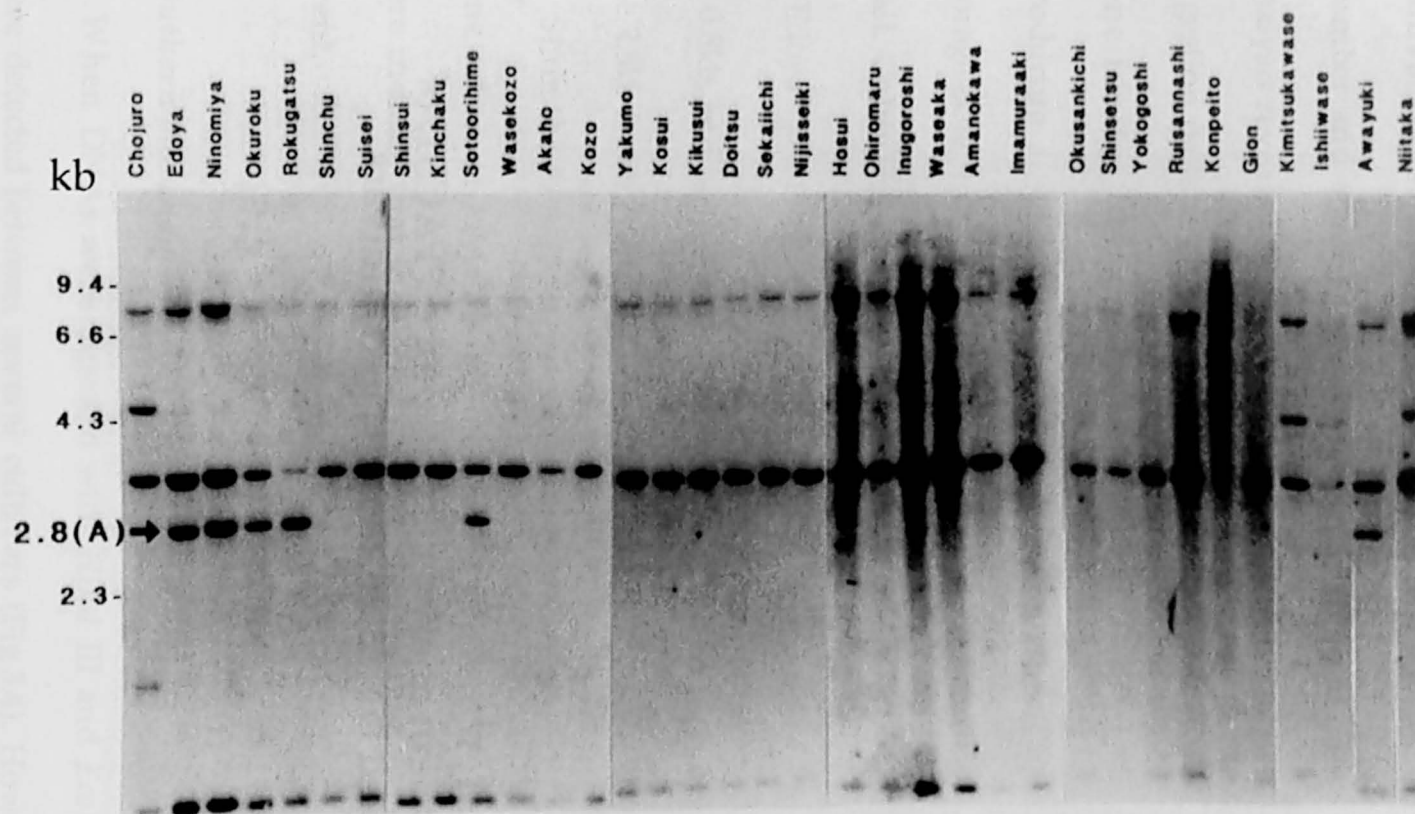


Fig. 3.1 Southern analysis, using probe PPACS1, of *Hind*III-digested DNAs from 35 Japanese pear cultivars. The band indicated by the arrow is specific to cultivars producing high levels of ethylene during fruit ripening.

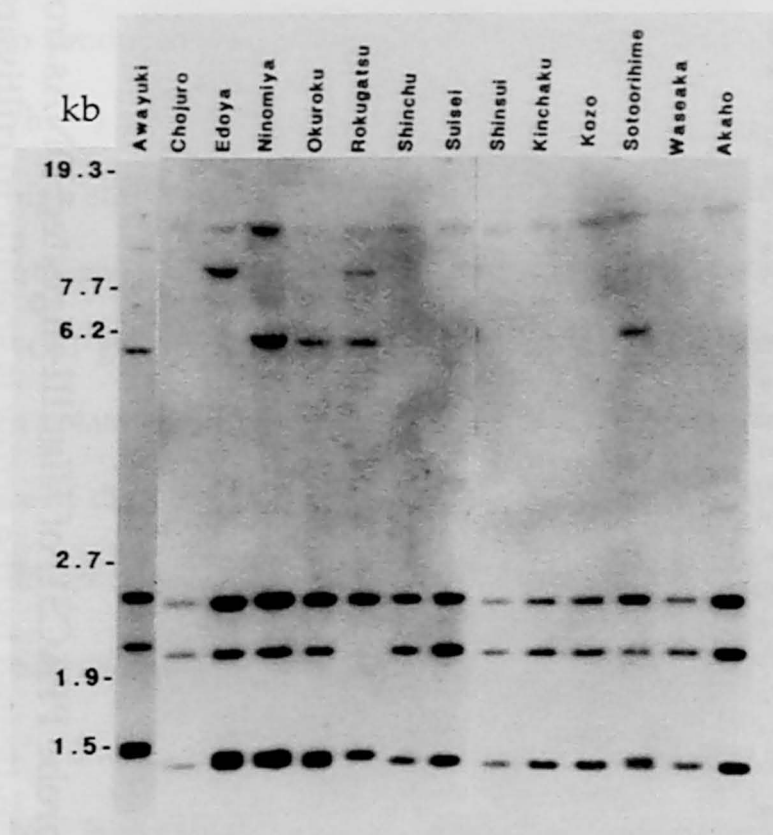


Fig. 3.2 Southern analysis, using probe PPACS1, of *Eco*RI-digested DNAs from indicated cultivars.

III band of 0.8kb. This band was absent in all cultivars showing high and low ethylene production, except for 'Ninomiya'. A survey of Japanese pear cultivars revealed a high level of polymorphism, as demonstrated by the number and size of bands more than 6.2kb in *Hind* III digests. Sequence analysis revealed that *PPACS2* has a *Hind* III site, and analysis of its restriction digestion pattern demonstrates that *PPACS2* belongs to a divergent multiple gene family. It is possible that the 0.8kb fragment is specific to cultivars producing a moderate level of ethylene. These cultivars also have poor storage potential, so the 0.8kb band may be a useful selective marker for this trait. Cultivars producing high levels of ethylene such as 'Rokugatsu', 'Edoya', 'Okuroku', 'Awayuki', and 'Sotoorihime' had no band of 0.8kb, and only 'Ninomiya' had both the 0.8kb fragment of *PPACS2* and the 2.8kb fragment of *PPACS1*.

Since the *PPACS1* gene produces more ethylene than that of *PPACS2*, the gene action of *PPACS1* will mask that of *PPACS2*. The findings presented here indicate that the *PPACS2* gene also resulted from a gene duplication event.

#### *Southern blot analysis using PPACS3 as a probe*

When DNAs were digested with *Hind* III and *Eco*RI, polymorphisms were detected between several cultivars (Fig.3.4). However, digestion with *Hind* III or *Eco*RI revealed identical patterns for almost all cultivars and no polymorphisms linked to ethylene production during fruit ripening were

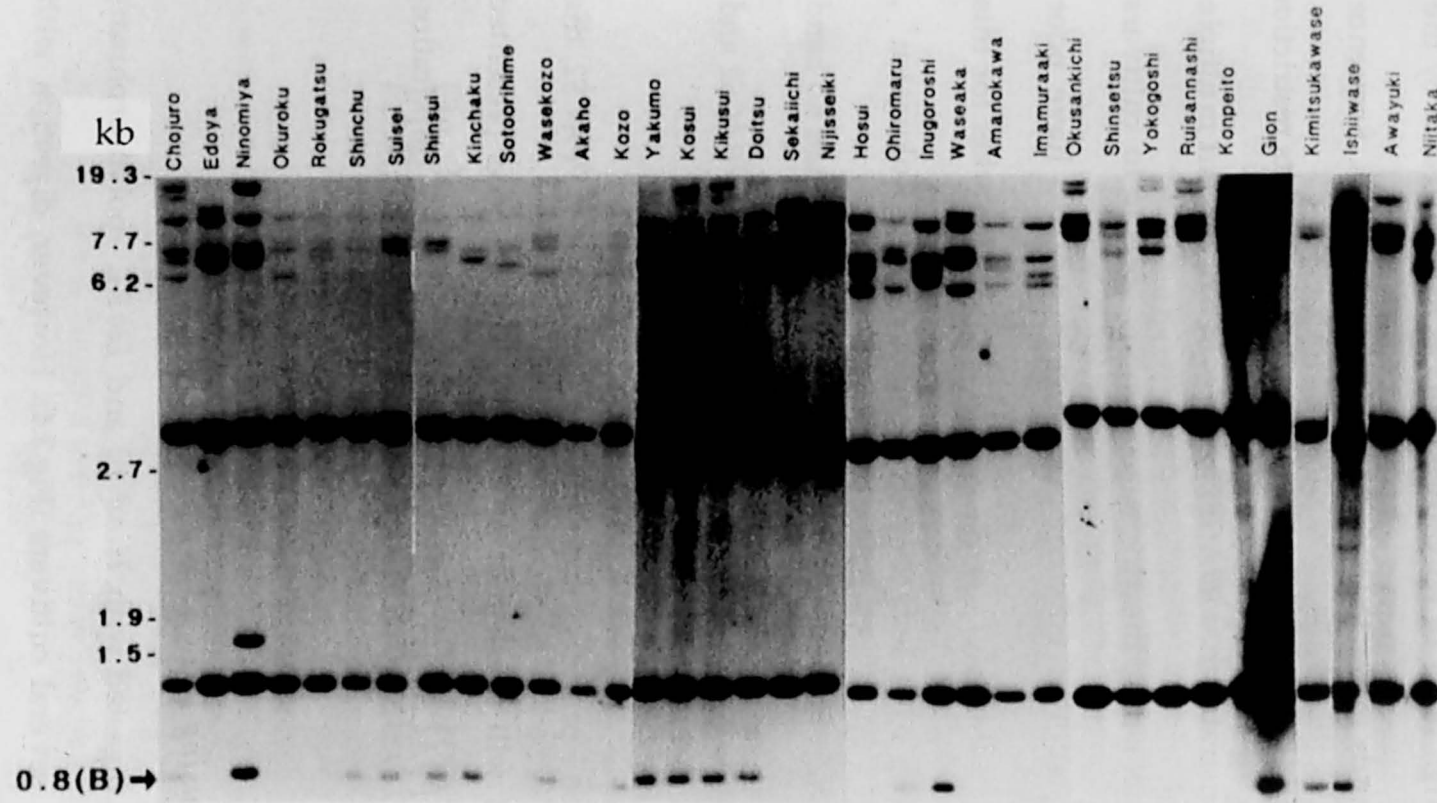


Fig. 3.3 Southern analysis, using probe PPACS2, of *Hind*III-digested DNAs from 35 Japanese pear cultivars. The band indicated by the arrow is specific to cultivars producing moderate levels of ethylene during fruit ripening.

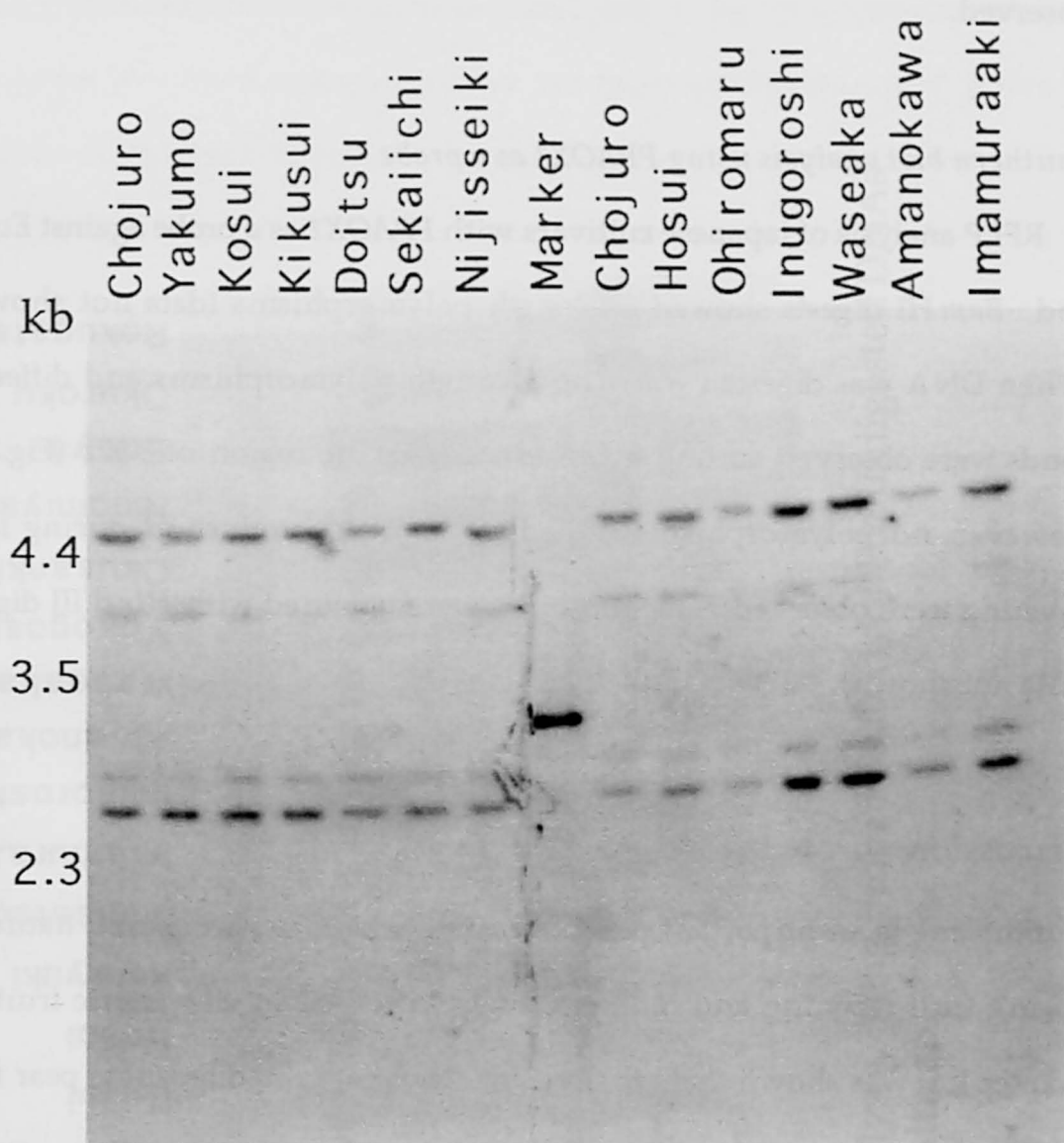


Fig. 3.4 Southern analysis, using probe PPACS3, of *Hind*III-digested DNAs from indicated cultivars.



observed.

#### *Southern blot analysis using PPAOX1 as a probe*

RFLP analysis of Japanese cultivars with *PPAOX1* as a probe against *EcoRV* and *Bam* HI digests showed no length polymorphisms (data not shown). When DNA was digested with *Eco* RI, length polymorphisms and different bands were observed among some cultivars in the region of 3.5kb (Fig.3.5). However, no polymorphisms linked to ethylene production during fruit ripening were observed. Similar results were obtained with *Hind* III digests (data not shown).

### **Discussion**

Ethylene is an important plant hormone which is synthesized naturally during fruit ripening and reduces storage potential in climacteric fruit. In chapter 1, it was shown that ethylene levels of cultivated Japanese pear fruit varied from 0.1 to 300  $\mu$ L C<sub>2</sub>H<sub>4</sub>/kg. f.w./hr. during ripening and that cultivars producing moderate or high levels (0.5 to 300  $\mu$  L C<sub>2</sub>H<sub>4</sub>/kg. f.w./hr.) generally have a poor storage potential (Kajiura and Sato, 1990). Since two enzymes (ACC synthase and ACC oxidase) are involved in ethylene biosynthesis, an interesting question is whether these genes are expressed during fruit ripening of Japanese pear. Three ACC synthase cDNAs (*PPACS1*, *PPACS2*, and *PPACS3*) and one ACC oxidase cDNA (*PPAOX1*) were isolated that operate in fruit ripening. As described in chapter 2, since

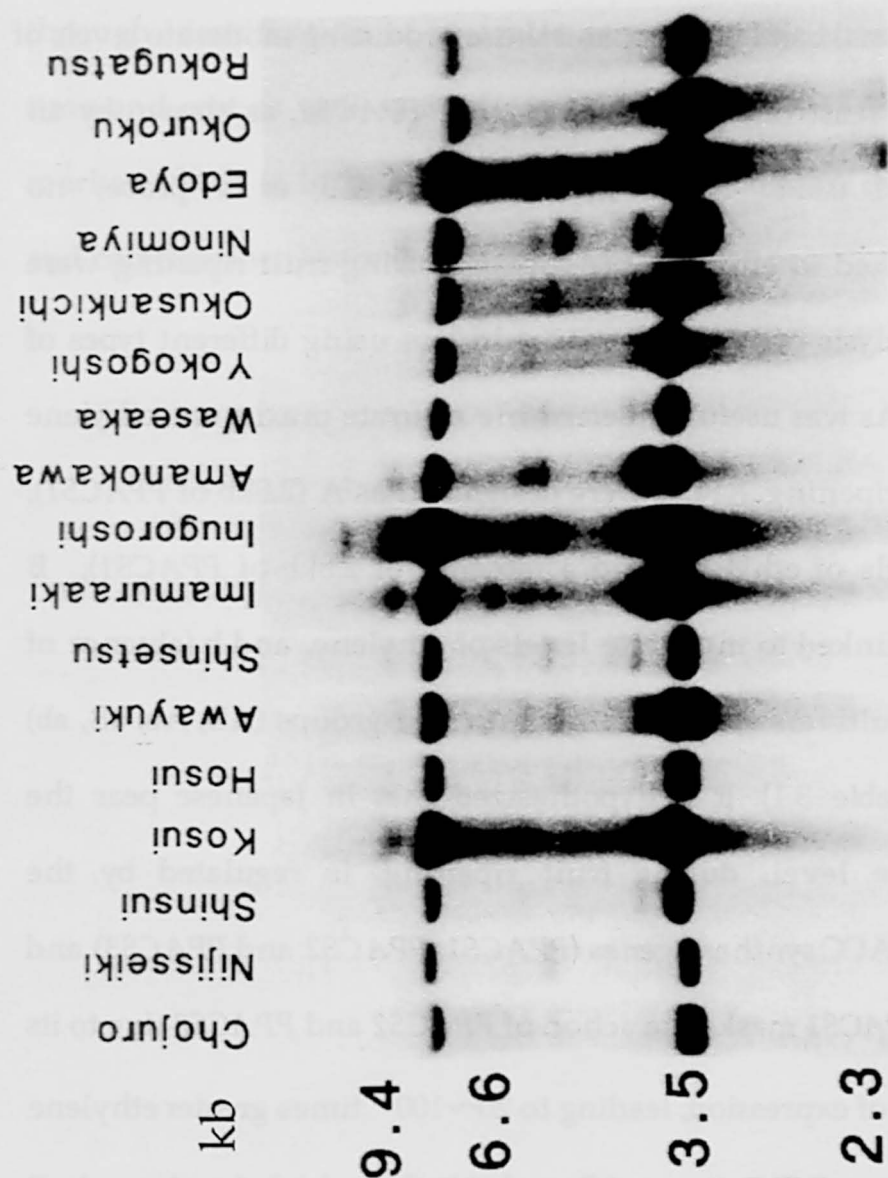


Fig. 3.5 Southern analysis, using probe PPAOX1, of *Eco*RI-digested DNAs from the indicated cultivars.

*PPAOX1* transcripts were highly expressed in all cultivars during fruit ripening, the ACC oxidase was not a limiting step in determining the maximum ethylene levels in Japanese pear cultivars. Cultivars producing high levels of ethylene possess at least one additional copy of *PPACS1*, as shown by an additional band of 2.8kb; and those producing moderate levels of ethylene possess at least one additional copy of *PPACS2*, as shown by an additional band of 0.8kb. However, using *PPACS3* as a probe, no polymorphisms linked to ethylene production during fruit ripening were observed. RFLP analysis of Japanese pear cultivars using different types of ACC synthase cDNAs was useful to determine accurate maximum ethylene levels during fruit ripening. RFLPs were designated as A (2.8kb of *PPACS1*), linked to high levels of ethylene, and a (absence of 2.8kb of *PPACS1*), B (0.8kb of *PPACS2*), linked to moderate levels of ethylene, and b (absence of 0.8kb of *PPACS2*). Cultivars were classified into four groups (AB, Ab, aB, ab) based on RFLPs (Table 3.1). It is hypothesized that in Japanese pear the maximum ethylene level, during fruit ripening, is regulated by the expression of three ACC synthase genes (*PPACS1*, *PPACS2* and *PPACS3*) and that the action of *PPACS1* masks the action of *PPACS2* and *PPACS3* due to its much higher levels of expression, leading to 20~100 times greater ethylene production. Therefore, RFLPs types AB and Ab show high levels and aB shows a moderate level of ethylene production during fruit ripening.

Although refrigeration systems have been developed, cultivation of cultivars with a short storage potential is limited and those having a long

storage potential are predominating. Progress in Japanese pear breeding is hindered by several factors, such as self-incompatibility, a long juvenile period and the requirement for large fields. The use of molecular markers could be useful to Japanese pear breeding by increasing selection efficiency through identification of favorable genetic combinations, thereby reducing time and cost as well as field space. In this study, I identified a molecular marker which is linked to high ethylene production during fruit ripening and correlates with storage potential in Japanese pear.

### 3.2 PCR-generated molecular markers for the 1-aminocyclopropane-1-carboxylate synthase genes regulating ethylene production during fruit ripening

#### Introduction

Previously, RFLP markers tightly linked to the locus conferring the ethylene evolution rate of ripening fruit were identified using RFLP analysis with two ACC synthase genes (*PPACS1* and *PPACS2*). RFLPs were designated as A (2.8kb of *PPACS1*), linked to high levels of ethylene ( $\geq 10 \mu\text{l/kg f.w./hr}$ ) and B (0.8kb of *PPACS2*), linked to moderate levels of ethylene ( $0.5 \mu\text{l/kg f.w./hr} \sim 10 \mu\text{l/kg f.w./hr}$ ), when the total DNA was digested by *HindIII*. It was proposed that in Japanese pear the maximum ethylene level, during fruit ripening, is regulated mainly by the expression of these two ACC synthase genes and that the action of A masks that of B due to its much higher levels of expression. Therefore, RFLPs of AB and Ab show high levels and aB shows moderate levels of ethylene synthesis during fruit ripening.

Molecular markers provide a powerful tool to understand the inheritance of certain traits, and they increase selection efficiency, especially in tree crops requiring long periods for breeding. It is possible that identified RFLP markers are applicable to marker assisted selection of seedlings with good storage potential in Japanese pear. Here the transformation of two RFLP markers into more convenient and easier PCR based markers is presented.

## Materials and methods

### *Plant materials*

Cultivars of Japanese pear (*Pyrus pyrifolia* Nakai) and Chinese pear (*P. bretschneideri* Rehd.) grown at the orchard of Tottori University were used in this study. Either young or mature leaves were used for DNA extraction. The extraction procedure was as described by Teramoto et al. (1994).

### *Cloning of 5' untranslated region of PPACS1*

The 5' flanking regions of *PPACS1* were obtained by the inverse PCR techniques. Genomic DNA (1  $\mu$ g) of 'Rokugatsu' was digested by *Hind*III, self-ligated by T4 ligase, and used as template for inverse PCR. Inverse PCR amplification of the 5' flanking region was performed by using *Taq* polymerase (Nippon Gene) and primers ACSINV1-1 (5'-GGCACCACAATGACCAGAAACG-3') and ACSINV1-2 (5'-GAGTCTTGGCCGTGAGAGTTGA-3'). The amplified fragments were purified, cloned into pGEM-T vector and sequenced.

### *PCR amplification of fragments specific to cultivars with high levels of ethylene during fruit ripening*

Based on sequencing data of 5' flanking regions of *PPACS1* (Fig. 3.6), forward (SYNP1-1: 5'-AACTCAAAATTTTCAAGCTA-3') and reverse (SYNP1-2: 5'-GATATTATCTCAAAATGACA-3') primers were designed to

amplify the fragments associated with high levels of ethylene during fruit ripening. The amplification reaction was performed in 50  $\mu$ l volumes and contained 100 ng of genomic DNA, 1 X *Taq* polymerase buffer, 50 pmol of primers, 0.2 mM each of the four dNTPs and 1.25 U *Taq* polymerase (Nippon Gene, Japan). The amplification was carried out in a TP-2000 thermal cycler (Takara Shuzo, Japan) programmed for 40 cycles of 60s at 94°C, 60s at 50°C and 90s at 72°C, then a final cycle at 72°C for 10 min. Amplified fragments were analyzed on 1.2% agarose gels in 1 X TAE buffer and stained by ethidium-bromide.

#### *PCR-RFLP analysis of cultivars with moderate levels of ethylene during fruit ripening*

Based on sequencing data of *PPACS2*, the following primers were synthesized: SYN3-1: 5'-GTCACAGAATCAACGATTGA-3' and SYN3-2: AGTAGAACGCGAAAACAAAT-3'. The PCR reaction was the same as mentioned above except for the program. The amplification program was 40 cycles of 60s at 94°C, 60s at 60°C and 120s at 72°C, then a final cycle at 72°C for 10 min. Amplified fragments were digested by *Hind*III and separated on 1.2% agarose gels in 1 X TAE buffer. After staining with ethidium bromide, gels were observed and photographed under UV light.

## **Results and discussion**

A fragment of 5' flanking regions of *PPACS1* was obtained by the inverse

PCR. The amplified fragment (2.0kb) was cloned and sequenced. This sequence contained 1.6kb of the 5' flanking regions of *PPACS1*, as shown in Fig. 3.6. Analysis of this sequence showed high identities (88%) with 5' flanking regions of apple ACC synthase gene (*MdACS1*) (Sunako et al., 1999). The coding region of *PPACS1* had 97% identity with that of *Md-ACS1*. This shows that a strong interspecific homology exists among ACC synthases and that the same expression mechanisms may exist.

Based on sequencing data of 5' flanking regions of *PPACS1*, SYN1-1 and SYN1-2 primers were designed. All available Japanese pear cultivars were tested by PCR with primers SYN1-1 and SYN1-2. Furthermore, 2 cultivars of *P. bretschneideri* ('Cili' and 'Yali') were used in the PCR assay. In Fig. 3.7, the 1.2kb product was specific to 8 cultivars ('Awayuki', 'Cili', 'Edoya', 'Ninomiya', 'Ninomiyahakuri', 'Okuroku', 'Rokugatsu' and 'Yali') which produced high levels of ethylene (more than 10  $\mu\text{L C}_2\text{H}_4/\text{kg. f.w./hr.}$ ) during fruit ripening. These findings coincide with the former Southern blot analysis using *PPACS1* as a probe. This PCR diagnosis therefore distinguishes high ethylene producers from moderate and low ethylene producers.

PCR amplification using primers specific for *PPACS2* produced a fragment of about 2.1kb that was amplified from all cultivars. After being digested with *HindIII*, the single PCR fragment was cleaved into several fragments of 1.2kb, 0.9kb, 0.8kb and 0.4kb (Fig. 3.8). Cultivars with moderate levels of ethylene during fruit ripening, for example cvs. Shinsui, Chojuro, Kosui, Kikusui and Yakumo, were characterized by the presence of 0.8kb and 0.4kb *HindIII*



10	20	30	40	50	60
CCGGTACTGT	TCACTTTAAC	AAAAAACCA	TATTTTTACA	TTAAAAAGTC	AATCCTGATA
70	80	90	100	110	120
CTATTCACCT	TATCCTTTAT	TTTGTCTTA	TCGTTAAAC	<u>TCAAAATTTT</u>	<u>CAAGCTATTT</u>
130	140	150	160	170	180
TCATTAATTT	TCCTTAACAA	AAATGGTTTT	ATTATAATAA	ATTATTCTAG	TGTTTTCCCT
190	200	210	220	230	240
GTTTTGTATA	CCTAATTATG	AAGGAGATAG	AGTGATGATG	TTAAATGAAG	AAAAAAGAGA
250	260	270	280	290	300
GATGCCATTT	TTTGTTTGTA	CCGGATTTTC	GAGGTTGACT	CGAATCAAAA	CATTGTTTGG
310	320	330	340	350	360
TAATTGGAGT	ACTGAGCAGA	CACAAAAACC	TGTACAAACT	TAAAGGTAA	AAAAAAAAG
370	380	390	400	410	420
TTTTAACGAA	AAGTTTTCAG	TATTGTTTAT	TTTAATAAAA	AATTATATTT	TTATATTAAA
430	440	450	460	470	480
AAGTTAATCC	TGGTACTGTT	TACTTTACCC	TTTATTTTGT	TTAAATTTCA	AATTTTTCAA
490	500	510	520	530	540
ATTTTTTTTCG	TTAATTTTTC	TTTGAAAAAA	AAAAAGAAAA	AAAAAGAAAA	ATACCCGACA
550	560	570	580	590	600
AAATTAGTTT	TGGCCACTAG	AGTCAAGAAG	CATATGGACC	AGGGTGGGTC	CCTTAGGCAT
610	620	630	640	650	660
TTTCTATGAT	ACTTGTGTGC	GGTAAGGTTT	TGTAACAAAA	ACTAGACCCG	AGTATTAATT
670	680	690	700	710	720
CTTGTITCTT	TGTTTTTTTT	CACATTAAAG	CCGATTAATG	CTTCTATGTA	CACTTGTAAT
730	740	750	760	770	780
CCCCACGCAA	GTTCTAGGT	AATGCCAGGT	AATGGTGAAC	GCCCTACCCA	CTTCGCAGTC
790	800	810	820	830	840
CAAGCAAATA	GTGAGAAAAT	AAATTAATGG	ATAATGGGAG	AAAAATTAAA	TTTAGAGATA
850	860	870	880	890	900
AAATTGTGTA	ACTAAATGAT	GTGTTATCAA	TAGGAATTAG	CACGTTTATC	AACGTTTAAG
910	920	930	940	950	960
TAATAAATCA	ATCATTAACT	TCCATATGAT	TTAGTTTCTA	AAATTTTATT	TACAAATTTA
970	980	990	1000	1010	1020
ACCTCTCTAG	TATTACTCTT	AATTAATGAT	TTAATGTTGA	TTAGTAACAA	CTAAAACTTC
1030	1040	1050	1060	1070	1080
ATTGCTTTGG	GATTGGGAA	TGTGTGAAGG	TCCTTCTTGA	TCAATGTCTT	TGCTTTGGAC
1090	1100	1110	1120	1130	1140
GGTGGAGCAA	AAGCGCGTAC	AATTATCATG	TTGTTTTTGG	ATTTTTATTG	AATCAAAATA
1150	1160	1170	1180	1190	1200
CTTTGATCAT	AATGTTAAGA	AAAAGAATCA	GAGAAATGCT	AAAGAGATTT	TCTTAAAGT
1210	1220	1230	1240	1250	1260
GAGATTTTTC	ATAATTTATT	TATCATGTTT	TTGGTACAAT	ATTTATAATG	CAGCCGCATA
1270	1280	1290	1300	1310	1320
AATTAATGTT	AAAATGTAAG	ATAACAGAGA	ATTCATAAAA	<u>AGTGTCAATT</u>	<u>TGAGATAATA</u>
1330	1340	1350	1360	1370	1380
<u>TCCTTATCAT</u>	<u>TTCTCAAAAA</u>	<u>ATATAACTAC</u>	<u>TCAGTGTGAC</u>	<u>GTGGCATTCC</u>	<u>TTTGTTAGAC</u>
1390	1400	1410	1420	1430	1440
AAATAATTTT	TATATATTTA	AATTTATATG	ATTACTTTTT	GCTATATATA	GACCCCTCCC
1450	1460	1470	1480	1490	1500
GTCCAACAAC	ATCCAATATC	CCACTTCAAA	CTTGTAATCC	AAAACCAAAC	CCTCAAATC
1510	1520	1530	1540	1550	1560
TCGCTCTATT	GCTTTCTCTT	CCTTCCACA	CTTCTGTCTT	ATAGCTTGTA	TCCATACAGA
1570	1580	1590			
AGAAAATTAA	CCAAAATGCG	CATGTTATCC	AGAAACGC		

**Fig.3.6** Nucleotide sequence of 5' region of *PPACS1* gene. The primer sequences are underlined.

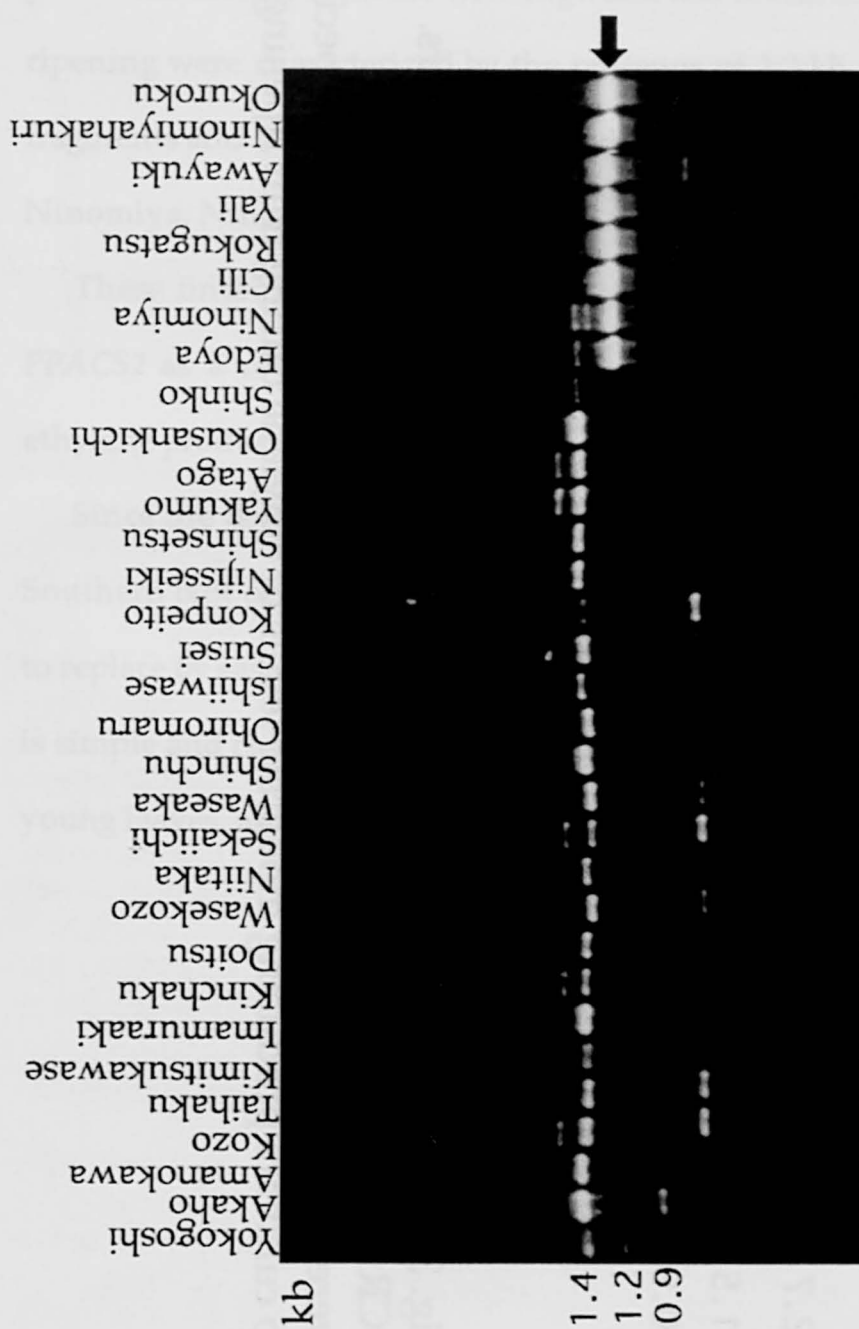


Fig. 3.7 PCR amplification for *PPACS1* gene from genomic DNAs of the indicated cultivars. The band indicated by the arrow is specific to cultivars producing high levels of ethylene during fruit ripening.

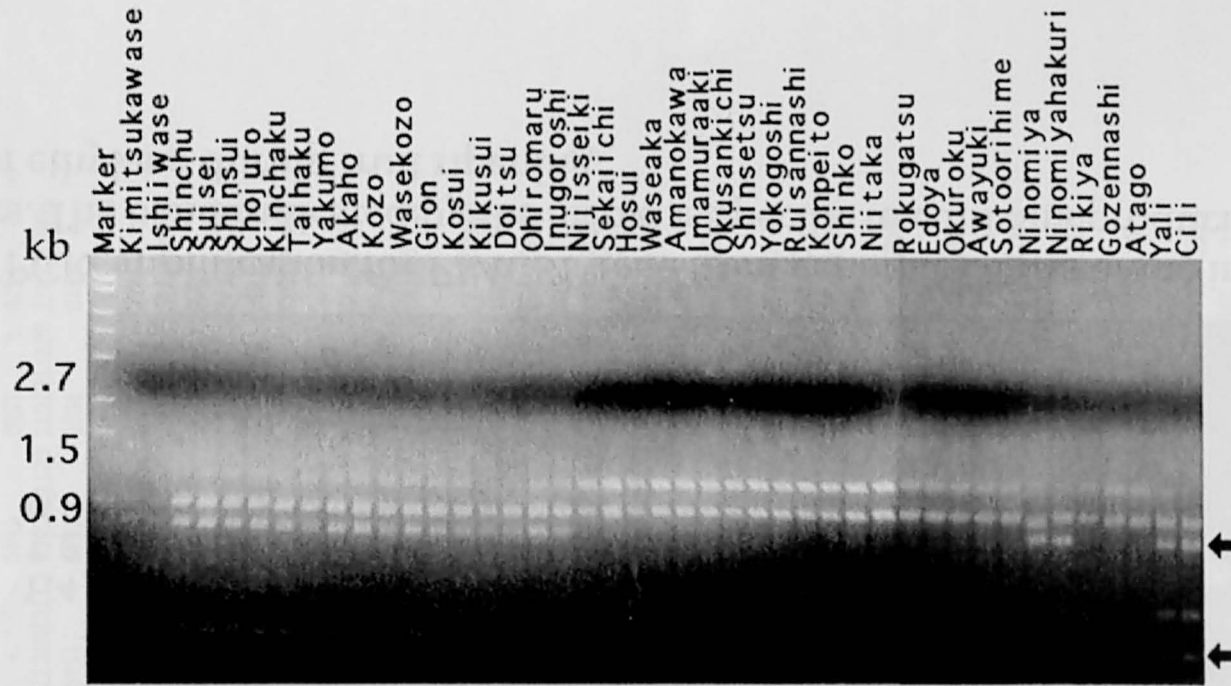


Fig. 3.8 PCR-RFLP analysis for *PPACS2* gene in the indicated cultivars. PCR products (2.1kbp) amplified with SYN3-1 and SYN3-2 primers were digested with *Hind*III. The bands indicated by the arrows are specific to cultivars producing moderate levels of ethylene during fruit ripening.

restriction fragments. This shows that a 1.2kb restriction fragment appears to generate the 0.8kb and 0.4kb restriction fragments in moderate ethylene producers. Hence, cultivars with high and low levels of ethylene during fruit ripening were characterized by the presence of 1.2 kb and 0.9 kb restriction fragments and lacks the 0.8kb and 0.4kb restriction fragments, except for cvs. Ninomiya, Ninomiyahakuri, Yali and Cili.

These findings coincide with the former Southern blot analysis using *PPACS2* as a probe. This PCR diagnosis therefore distinguishes moderate ethylene producers from high and low level ethylene producers.

Since the determination of the storage potential by progeny analysis for Southern blot of two ACC synthases is a time-consuming task, it is desirable to replace by easier methods based on PCR. This PCR diagnosis described here is simple and requires only small amounts of DNA, preferably obtained from young leaves, as a starting material.

### **3.3 Determination of genotypes of Japanese pear cultivars in relation to ethylene production and the mode of inheritance of ethylene production during fruit ripening.**

#### **Introduction**

Using the gene diagnosis described in the previous section, I identified four RFLP types (AB, Ab, aB, ab) based on RFLPs. RFLP types AB and Ab showed high levels and aB showed a moderate level of ethylene production during fruit ripening.

However, since the identified RFLP markers are dominant markers, the allele-specific PCR-based methods cannot distinguish dominant homozygous genotype from heterozygous genotype. Therefore, test crosses are needed to identify the heterozygosity. In this section, the allele-specific PCR-based method was applied to test cross lines to determine the ethylene synthetic genotypes of Japanese pear cultivars that are commercially important and used in breeding.

#### **Materials and methods**

Eight progenies were used. These crosses are listed in Table 3.2. 'Rokugatsu' fruit produces high levels of ethylene ( $\geq 10 \mu\text{l/kg f.w./hr}$ ) (RFLP type: Ab); 'Yakumo', 'Shinsui', 'Chojuro' and 'Kosui' fruit produce moderate levels of ethylene ( $0.5 \mu\text{l/kg f.w./hr} \sim 10 \mu\text{l/kg f.w./hr}$ ) (RFLP

type: aB); 'Nijisseiki', 'Osanijisseiki' (a self-compatible mutant of 'Nijisseiki'), 'Hosui' and 'Atago' fruit produce low levels of ethylene ( $<0.5 \mu\text{L/kg/hr}$ ) (RFLP type: ab); 'Yali' and 'Cili' fruit produce high levels of ethylene (RFLP type: AB). Either young or mature leaves were used for DNA extraction. The extraction procedure was as described by Teramoto et al. (1994).

PCR amplification and PCR-RFLP analysis were the same as described in chapter 3.2.

## Results and discussion

The segregation of 'Osanijisseiki' (ab) X 'Kosui' (aB) population fitted a ratio of 1:1 for aB and ab (Table 3.2). This result indicated that 'Kosui' was heterozygous for B and that the genotype of 'Kosui' was aaBb (Table 3.3). The segregation of 'Osanijisseiki' (ab) X 'Shinsui' (aB) population fitted a ratio of 1:0 for aB and ab (Table 3.2). This indicated that 'Shinsui' was homozygous for B and that the genotype of 'Shinsui' was aaBB (Table 3.3). 'Shinsui' is an early maturing cultivar (harvested at the beginning of August), important as a breeding material. However, if the objective of breeding is storage ability in Japanese pear, using 'Shinsui' as a parent is inadequate because all the progenies of 'Shinsui' are predicted to have a low storage potential. This was confirmed by the fact that all the newly bred cultivars using 'Shinsui' as a parent, 'Wakahikari' ('Shinsui' X 'Hosui') and 'Natsuhikari' ('Shinsui' X 'Chosui') both from Chiba prefecture and 'Akemizu' ('Shinsui' X '42-6' ('Kumoi' X 'Kosui')) from Kanagawa prefecture were early maturing

Table 3.2 Segregation analysis for *PPACS1* and *PPACS2* genes in eight progenies

Parentage (RFLP type)	No. of plants tested	Segregation of RFLP type AB:Ab:aB:ab	Expected ratio	Chi-square	Probability
Osanijisseiki (ab) X Kosui (aB)	27	0 : 0 : 13 : 14	1 aB : 1 ab	0.04	0.75<P<0.9
Osanijisseiki (ab) X Shinsui (aB)	17	0 : 0 : 17 : 0	1 aB : 0 ab	0	1
Yakumo (aB) X Rokugatsu (Ab)	25	14 : 11 : 0 : 0	1 AB : 1 Ab	0.36	0.5<P<0.75
Nijisseiki (ab) X Chojuro (aB)	30	0 : 0 : 8 : 22	1 aB : 1 ab	6.53	0.01<P<0.025
Osanijisseiki (ab) X Cili (AB)	24	9 : 0 : 15 : 0	1 AB : 1 aB	1.5	0.1<P<0.25
Nijisseiki (ab) X Yali (AB)	41	23 : 18 : 0 : 0	1 AB : 1 Ab	0.61	0.25<P<0.5
Osanijisseiki (ab) X Atago (ab)	24	0 : 0 : 0 : 24	0 aB : 1 ab	0	1
Osanijisseiki (ab) X Hosui (ab)	24	0 : 0 : 0 : 24	0 aB : 1 ab	0	1

cultivars (harvested at August) and had a low storage potential (Kotobuki, 1997).

The segregation of 'Yakumo' (aB) X 'Rokugatsu' (Ab) population fitted a ratio of 1:1 for AB and Ab (Table 3.2). This suggested that 'Rokugatsu' was homozygous for A and 'Yakumo' was heterozygous for B and that the genotype of 'Rokugatsu' was AAbb and that of 'Yakumo' was aaBb (Table 3.3).

In 'Nijisseiki' (ab) X 'Chojuro' (aB) populations, of 30 seedlings, 8 were aB and 22 were ab (Table 3.2). Thus, progenies did not fit the expected 1 aB : 1 ab ratio. However, the genotype of 'Chojuro' was predicted to be aaBb. In this combination, the segregation distortion of resistance to black spot disease was reported (Sanada et al., 1994). The excess of ab seedlings could be because *PPACS2* (B) is linked to lethal genes or because aB seedlings have low viability.

Two progenies ('Osanijisseiki' X 'Cili' and 'Nijisseiki' X 'Yali') were both ab x AB populations. Progenies of 'Osanijisseiki' X 'Cili' fitted the expected 1 AB : 1 aB ratio (Table 3.2), while progenies of 'Nijisseiki' X 'Yali' fitted the expected 1 AB : 1 Ab ratio (Table 3.2). These findings suggested that the genotype of 'Cili' was AbBB and that of 'Yali' was AABb (Table 3.3).

In two progenies ('Osanijisseiki' X 'Atago' and 'Osanijisseiki' X 'Hosui'), both were ab X ab for *PPACS1* and *PPACS2*. All the seedlings analyzed were ab RFLP types (Table 3.2). This result was a natural consequence.

In conclusion, the genotypes of ethylene production were determined in several cultivars. This information is useful for breeding strategies to



Table 3.3 Identification of genotypes for *PPACS1* and *PPACS2* genes in several cultivars derived from the results of Table 3.2

Cultivars	Predicted genotype
Atago	<i>aabb</i>
Chojuro	<i>aaBb</i>
Cili	<i>AaBB</i>
Hosui	<i>aabb</i>
Kosui	<i>aaBb</i>
Nijisseiki	<i>aabb</i>
Osanijisseiki	<i>aabb</i>
Rokugatsu	<i>AAbb</i>
Shinsui	<i>aaBB</i>
Yakumo	<i>aaBb</i>
Yali	<i>AABb</i>

improve the storage ability in Japanese pear.

## Chapter 4.

# Isolation of fruit ripening related genes and possible involvement of ethylene in their expression

### Introduction

As described in the previous chapters, there is a large varietical difference in ethylene synthesis during fruit ripening in Japanese pear. It is generally thought that ethylene production affects many ripening processes. The physiological and biochemical changes which occur during fruit ripening result from *de novo* protein synthesis and changes in the activities of various enzymes, which are regulated by differential gene expression and mRNA abundance. Ethylene is known to exert its effects, at least in part, by altering gene expression. Effects on both transcriptional and post-transcriptional processes have been identified (Deikman, 1997). A useful approach to investigate such changes is to isolate the transcripts encoding proteins associated with the ripening process, using differential screening or differential display techniques. In this chapter, the differences in gene expression between unripe and ripe Japanese pear fruit are described. Fruit ripening is considered to have both ethylene-dependent and ethylene-independent processes (Lelievre et al., 1997a). This chapter focuses on ethylene control of gene expression during fruit ripening. Using 1-methylcyclopropene (MCP), newly reported to be an inhibitor of ethylene action (Sisler and Serek, 1997), the regulation of isolated genes has been

classified here into either ethylene-dependent or ethylene-independent processes. Identification of ethylene-inducible genes in Japanese pear fruit can be very useful to understand the molecular nature of fruit ripening.

## **Materials and methods**

### *Plant material*

Japanese pear cvs. Nijisseiki and Kikusui fruits were used in this experiment.

### *Cloning and characterization of fruit ripening related genes by differential display*

Total RNA was extracted from the unripe (120DAF: Days After Flowering), ripe (153DAF: commercial harvest) and overripe stages (23DAH: Days After Harvest kept at 20°C) of 'Nijisseiki' fruits. A simple mRNA differential display method reported by Yoshida et al., (1994) was used. cDNA was synthesized, with an AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Science), using random hexamer primers from 0.5  $\mu$ g of Poly(A)<sup>+</sup>RNA. PCR was performed using a single 10-mer RAPD primer (OP26-1 to OP26-26, Operon Technologies, USA) for each reaction. The PCR products were separated by electrophoresis on 3.5% polyacrylamide gels and visualized by ethidium bromide staining. Differentially displayed bands were cut out and cDNA fragments specific to ripe and overripe fruits were re-amplified, purified and cloned into pGEM T-Vector (promega). Five  $\mu$ g of

poly(A)<sup>+</sup> RNA from overripe fruit (23DAH) of 'Nijisseiki' was used to construct a cDNA library of 200,000 clones. Cloned specific fragments were <sup>32</sup>P-labeled by the random primed method (Amersham) and used to screen the cDNA library. Screened cDNA clones were sequenced and database analysis was carried out using BLASTN or TBLASTN programs.

For Northern analysis, RNA was prepared from fruit at various stages of development (53DAF and 82DAF), and other organs (immature leaves, mature leaves, yellowing leaves, dormant buds, current shoots). Conditions for Northern analysis was the same as described in chapter 2.

#### *Cloning and characterization of fruit ripening related genes by differential screening*

Unripe Japanese pear fruit (cv. Kikusui) were harvested at 132DAF from the orchard of Tottori University. Fruits were kept at 20°C. Total RNA was extracted from the unripe (132DAF) and ripe (14DAH) fruit. The ripe fruit cDNA library was constructed with an efficiency of  $0.9 \times 10^6$  pfu per microgram of cDNA. For library screening, one filter was hybridized to radiolabeled cDNA from unripe fruit, and the other one was hybridized to radiolabeled cDNA from ripe fruit. A total of  $1 \times 10^4$  plaques were screened. Thirty putative ripening-related cDNA clones selected from the library screening were sequenced. The sequences obtained were compared with all known DNA sequences using BLASTN or TBLASTN programs. Northern analysis of transcript levels during fruit ripening were determined by <sup>32</sup>P labeled isolated

cDNAs and ACC oxidase cDNA (*PPAOX1*) as probes with a FLA2000 Bio Imaging Analyzer (Fuji Film, Tokyo).

*Effect of 1-methylcyclopropene (MCP) on the expression of ripening related genes by differential display or differential screening*

MCP was synthesized according to the method of Sisler and Serek (1997).

‘Kikusui’ fruits (4DAH) were treated with 1 to 2 ppm MCP in 3L jars for 12hr and then allowed to ripen at 20°C for 2 days. Control fruits were 6 DAH fruits. Total RNA was isolated from control and MCP-treated fruits using hot borate methods. Probes for Northern analysis were cDNAs isolated by differential display or differential screening. The effect of MCP on the expression was determined by <sup>32</sup>P labeled isolated cDNAs and ACC oxidase cDNA (*PPAOX1*) as probes with FLA2000 Bio Imaging Analyzer (Fuji Film, Tokyo).

## **Results and discussion**

*Cloning and characterization of fruit ripening related genes by differential display*

cDNAs synthesized from unripe, ripe and overripe fruit RNA were used for differential display. The clones from ripe or overripe specific bands, JPR26-5 (329bp), JPR26-8 (118bp) and JPR26-24 (416bp), were obtained using OP26-5, OP26-8, and OP26-24 primers, respectively. Using JPR26-5, JPR26-8 and JPR26-24 as probes, *JPRORF1*, *JPROMT* and *JPRXYL* were isolated,

respectively. *JPRORF1* showed 29% homology with human dihydropyrimidine dehydrogenase (DPD) at the amino acid level (Yokota et al., 1994) (Table 4.1). The expression of *JPRORF1* was up-regulated during fruit ripening and observed in yellowing leaves (Fig. 4.1). This suggests that *JPRORF1* is a senescence-related gene. *JPROMT* showed 54% homology to the O-methyltransferase (OMT) from apricot (Mbeuie-A-Mbeguie et al., 1997) at the amino acid level. *JPROMT* transcripts accumulated to much higher levels in overripe fruit, expression being fruit specific (Fig. 4.1). This has also been observed for apricot OMT (Mbeuie-A-Mbeguie et al., 1997). The most interesting cDNA that I cloned, in terms of its potential function, is *JPRXYL*. The protein was shown to have a hydrophobic region of 23 amino acids (IAKLSLLSLLFLFSSLCSMAVVH) at the N-terminus which resembled a signal sequence as determined by the SOSUI and PSORT programs (Nakai and Kanehisa, 1992). Searches through the GenBank and SwissProt databases revealed that *JPRXYL* had similarities to  $\beta$ -D-xylosidase with the highest sequence identities being 33% for  $\beta$ -D-xylosidase of *Trichoderma reesei* (62% similarity) (Margolles-Clark et al., 1996).  $\beta$ -D-xylosidase hydrolyzes the  $\beta$ -1,4-D-xylopyranosyl linkage from xylan in hemicellulose and is concerned with the degradation of cell walls which results in fruit softening. The presence of xylan in pear fruit was demonstrated by Chanda et al. (1951). Yamaki and Kakiuchi (1979) showed that the activity of  $\beta$ -D-xylosidase increased with ripening and overripening in Japanese pear and the activity of the bound form was much higher than that of the soluble one. The deduced

**Table 4.1 Characterization of ripening-related cDNA clones isolated by differential display**

Clone	Accession No. for Pear clone	Transcript size (kb)	Homology to	Genbank Accession No.	Identity (%)
JPRORF1	AB014457	1.7	Dihydropyrimidine dehydrogenase ( <i>Homo sapiens</i> )	U09178	29
JPROMT	AB014456	1.5	O-methyltransferase ( <i>Prunus armeniaca</i> )	U82011	54
JPRXYL	AB007121	2.7	$\beta$ -D-xylosidase ( <i>Trichoderma reesei</i> )	Z69257	33



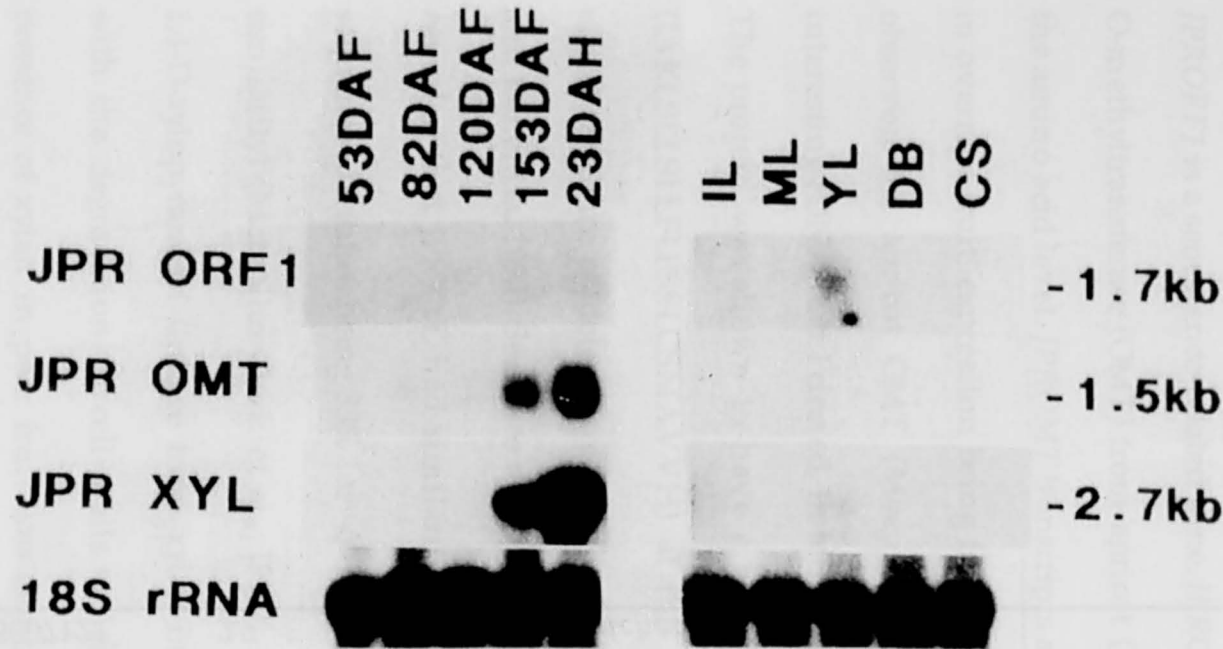


Fig. 4.1 Expression of *JPRORF1*, *JPROMT* and *JPRXYL* during fruit development, ripening, over-ripening and in different organs in Japanese pear cv. Nijisseiki. Total RNA ( $10\mu\text{g}$ ) was used for Northern blot analysis. The dots were probed with *JPRORF1*, *JPROMT*, *JPRXYL*, and 18S rDNA to estimate RNA loadings. The RNA samples were: 53DAF (Days After Flowering), 82DAF, 120DAF, 153DAF, 23DAH (Days kept at  $20^{\circ}\text{C}$  After Harvest), IL: immature leaves (partially expanded), ML: mature leaves (fully expanded), YL: yellowing leaves, DB: dormant flower buds and CS: current shoots.

amino acid sequence for *JPRXYL* gene includes a putative extracellular signal sequence of 23 amino acids. The transcript of *JPRXYL* increased during ripening and overripening in Japanese pear fruit (Fig. 4.1). These results suggest that *JPRXYL* encodes a cell wall bound  $\beta$ -D-xylosidase, which degrades xylan or arabinoxylan in hemicellulose cell walls leading to flesh breakdown during overripening in Japanese pear fruit.

#### *Cloning and characterization of fruit ripening related genes by differential screening*

Thirty differentially expressed clones that were selected by screening the ripe fruit library were classified into 11 nonredundant groups of cDNAs (Table 4.2). Identified up-regulated cDNAs are mainly associated with pathogenesis (*PPFRU* 9 and 19), protein metabolism (*PPFRU* 7, 11 and 32) or stress responses (*PPFRU* 8, 13, 16 and 21). Transcripts encoding two MT-like proteins (*PPFRU* 8 and 16) were abundant in Japanese pear during fruit ripening (Fig. 4.2). *PPFRU* 8 shared 78% identity with *PPFRU* 16. Clones encoding MT-like proteins have been isolated from developing kiwifruit, ripening banana fruit, cold-stored apple fruit, and developing satsuma mandarin fruit (Clendennen and May, 1997; Ledger and Gardner, 1994; Moriguchi et al., 1998; Reid and Ross, 1997), although their precise function in fruit development and ripening is unknown.

*PPFRU* 13 shared the highest sequence homology with the glycine-rich protein from *Arabidopsis thaliana* (Lang and Palva, 1992). *PPFRU* 21 shared

**Table 4.2 Characterization of up-regulated cDNA clones isolated by differential screening**

Clone	Accession No. for Pear Clone	No. of Clones	Transcript size (kb)	Homology to	High Score [P(N)]	GenBank Accession No. <sup>a</sup>
PPFRU7	AB021784	1	1.9	Cysteine protease ( <i>Pisum sativum</i> )	584[2.3e-75]	U44947
PPFRU8	AB021785	5	0.6	Metallothionein-like protein ( <i>Malus domestica</i> )	1372[6.4e-190]	U61973
PPFRU9	AB021786	4	1.1	Endo-chitinase class III ( <i>Beta vulgaris</i> )	860[8.7e-96]	S66038
PPFRU11	AB021787	1	1.8	Aspartic endopeptidase ( <i>Cucurbita pepo</i> )	2174[3.2e-174]	AB002695
PPFRU13	AB021788	9	0.8	Glycine rich protein( <i>Arabidopsis thaliana</i> )	261[3.7e-12]	L04173
PPFRU14	AB021789	1	0.9	Calmodulin ( <i>Cicer arietinum</i> )	381[1.2e-39]	Y09853
PPFRU16	AB021790	2	0.6	Metallothionein-like protein ( <i>Actinidia deliciosa</i> )	360[1.1e-21]	L27813
PPFRU19	AB021791	1	1.0	Polygalacturonase inhibitor ( <i>Pyrus communis</i> )	1760[5.8e-283]	L09264
PPFRU21	AB021792	4	0.9	Abscisic stress ripening protein ( <i>Solanum chacoense</i> )	732[1.3e-151]	U12439
PPFRU32	AB021793	1	2.1	Asparagine synthetase ( <i>Oryza sativa</i> )	272[3.3e-12]	U55873
PPFRU36	AB021794	1	1.0	F1-ATPase ( <i>Pisum sativum</i> )	694[3.5e-48]	L13320

<sup>a</sup>Most similar sequence as identified by BLASTN or TBLASTN searches.

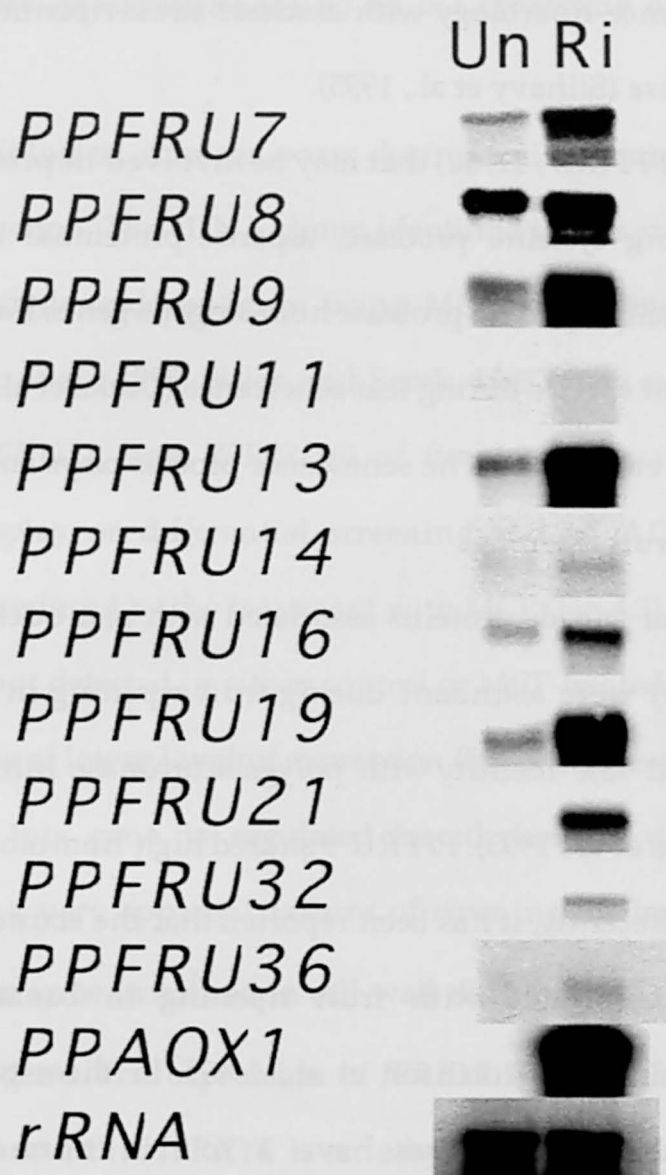


Fig. 4.2 Northern blot analysis of ripening-related clones during fruit ripening in 'Kikusui' pear. Total RNA was extracted from unripe and ripe fruits and each lane contained 10  $\mu$ g of total RNA. The RNA blots were hybridized with the radio-labeled inserts of the indicated cDNA clones. An rDNA probe was used to estimate RNA loadings and a Japanese pear cDNA clone for ACC oxidase was used as a ripening indicator. Un, unripe fruit (at harvest); Ri, ripe fruit (14 days after harvest).

the highest sequence homology with abscissic stress-ripening protein from *Solanum chacoense* (Silhavy et al., 1995).

Other clones (*PPFRU*7, 11, 32) that may be involved in protein metabolism are those encoding cysteine protease, aspartic proteinase and asparagine synthetase. Especially, cysteine protease homologous genes have been cloned from various plant species during leaf senescence (Drake et al., 1996; Lohman et al., 1994; Smart et al., 1995). The senescence process plays an important role in Japanese pear fruit ripening.

Transcripts that encode proteins associated with plant defense responses (*PPFRU* 9 and 19) were abundant during fruit ripening in Japanese pear. *PPFRU* 19 showed 98% identity with polygalacturonase inhibitor (PGIP) of Bartlett pear (Stotz et al., 1993). *PPFRU* 9 shared high homology with class III acidic chitinases. Recently, it has been reported that the abundant expression of chitinases is associated with fruit ripening in banana and grapes (Clendennen et al., 1998; Robinson et al., 1997). In these two reports, the authors indicated that chitinases have a role in normal growth and development during fruit ripening or have a role as a storage protein rather than pathogen-related functions. In Japanese pear fruit, *PPFRU* 9 protein may actually have functions other than pathogen resistance.

Other clones (*PPFRU* 14, and 36) showed high homology with calmodulin and F<sub>1</sub>-ATPase, respectively (Morikami et al., 1992; Nicolas et al., 1998; Okamoto et al., 1995). The expression of these clones was up-regulated during ripening, but the expression levels were lower than those of other clones.

### *Effect of 1-methylcyclopropene (MCP) on the expression of ripening related genes*

Many physiological changes occur during fruit ripening in response to ethylene production. The cDNA clones identified in this study were classified according to ethylene-dependence using MCP, which blocks the ethylene receptor (Serek et al., 1995; Sisler and Serek, 1997). The expression of three (*PPFRU16*, *PPFRU21*, and *PPFRU36*) of the 14 clones isolated by either differential display or differential screening and an ACC oxidase cDNA (*PPAOX1*) was reduced by the treatment with MCP (Fig.4.3). The expression of *JPRORF1* was not detected in either control or MCP-treated fruits by Northern analysis because of lower level of expression (Fig.4.4). Therefore, whether the expression of this gene is regulated by ethylene is unclear. Cell wall disassembly is a very apparent feature of ripening in fleshy fruit. A clone (*JPRXYL*) that may be involved in cell wall disassembly is encoded by  $\beta$ -D-xylosidase-like gene. The expression of *JPRXYL* was not affected by the MCP treatment. It shows that *JPRXYL* is an ethylene independent gene and that hemicellulose disassembly may be regulated by ethylene independent processes. The expression of ACC oxidase gene is considered to be subject to positive feedback regulation (Bouquin et al., 1997; Lelievre et al., 1997b; Nakatsuka et al., 1997). This implies that MCP affects the expression of some ripening-related gene. The evidence presented in this report demonstrates that the expression of *PPFRU16*, *PPFRU21* and *PPFRU36* is under positive feedback regulation by ethylene during fruit ripening. Endochitinase and



Fig. 4.3 Effect of MCP on the expression of ripening-related clones isolated by differential screening. Fruits (4days after harvest) were treated with (+) or without (-) 1 to 2 ppm MCP for 12hr and then ripened at 20°C for 2 days.

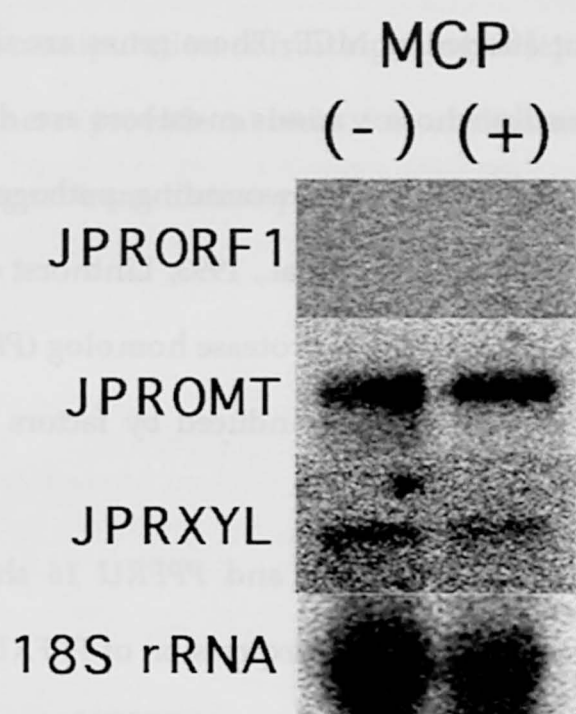


Fig. 4.4 Effect of MCP on the expression of ripening-related clones isolated by differential display. Fruits (4 days after harvest) were treated with (+) or without (-) 1 to 2 ppm MCP for 12 hr and then ripened at 20°C for 2 days.



cysteine protease mRNAs are known to be induced by ethylene (Cervantes et al., 1994; Kirsch et al., 1993; Pechan et al., 1999; Shinshi et al., 1995). However, the expression of cysteine protease homolog (*PPFRU7*) and endochitinase homolog (*PPFRU9*) was not affected by MCP. These genes are shown to be encoded by a multigene family whose various members are differentially expressed in response to many factors such as wounding, pathogen attack and drought (Dalisay and Kuc, 1995; Koizumi et al., 1993; Linthorst et al., 1993). This shows that a ripening-related cysteine protease homolog (*PPFRU7*) and endochitinase homolog (*PPFRU9*) may be induced by factors other than ethylene.

As previously described, both *PPFRU 8* and *PPFRU 16* showed high identity with type-2 MT-like protein, but the expression of *PPFRU8* was only developmentally regulated, while the expression of *PPFRU16* was regulated by the ethylene signal transduction pathway. A gene encoding a MT-like protein was shown to be expressed in leaf abscission zones of *Sambucus nigra* and promoted strongly by ethylene (Coupe et al., 1995). These findings show that plant MT gene expression can be up-regulated by ethylene. As described above, *PPFRU 21* shared the highest sequence homology with abscissic stress-ripening protein and *PPFRU36* showed high homology with F<sub>1</sub>-ATPase. This is the first indication that the expression of genes encoding abscissic stress-ripening protein and F<sub>1</sub>-ATPase can be regulated by ethylene. The expression of these genes did not disappear by the MCP treatment. These findings imply that their expression is regulated not only by ethylene, but also developmentally or by other factors. However, these three genes may be good

candidates determining the promoter sequence required for the ethylene response during fruit ripening. These regulatory elements will allow for spatial and temporal control of foreign protein expression and will facilitate the genetic manipulation of fruit ripening in Japanese pear. The cDNA clones described here provide a basis for understanding the involvement of ethylene signal transduction pathway in fruit ripening of Japanese pear.

## Summary

### Chapter 1 Cultivar differences in the ethylene production during fruit ripening

Ethylene production during fruit ripening was measured in existing cultivars in an effort to clarify the ripening characteristics of Japanese pear. The ethylene level in cultivated Japanese pear fruit was shown to vary from 0.1 to 300  $\mu\text{l C}_2\text{H}_4/\text{kg. f.w./hr.}$  during ripening. Every Japanese pear cultivar showed a characteristic pattern of ethylene evolution, some having a rapid increase in ethylene production and others having no or very little ethylene production. It was demonstrated that high and moderate ethylene producers tended to be early- or mid-maturing cultivars and low ethylene producers tended to be mid- or late-maturing cultivars.

### Chapter 2 Isolation and expression analysis of ethylene biosynthetic genes

Genes involved in the ethylene biosynthetic pathway were isolated to elucidate the cause of the differences in ethylene production with the cultivar. Three ACC synthase genes (*PPACS1*, *PPACS2*, *PPACS3*) and one ACC oxidase gene (*PPAOX1*) were isolated. *PPACS1* was shown to be specifically expressed in the cultivars with a high ethylene production level ( $\geq 10 \mu\text{l/kg f.w./hr}$ ) and *PPACS2* was shown to be specifically expressed in the cultivars

with moderate ethylene production level ( $0.5 \mu\text{l/kg f.w./hr} \sim 10 \mu\text{l/kg f.w./hr}$ ) during fruit ripening. The *PPACS3* transcript was expressed in all cultivars during fruit ripening regardless of the difference in the amount of ethylene synthesis with the cultivar. The *PPAOX1* transcripts were detected abundantly in all cultivars, regardless of whether the cultivars produced ethylene or not. This suggested that the ACC oxidase is not a limiting step in the ethylene biosynthetic pathway of Japanese pear during fruit ripening.

### **Chapter 3 The mode of inheritance and development of the gene diagnosis of ethylene production during fruit ripening**

RFLP markers tightly linked to the locus conferring the ethylene evolution rate of ripening fruit in Japanese pear were identified, using RFLP analysis with two ACC synthase genes (*PPACS1* and *PPACS2*). RFLPs were designated as A (2.8kb of *PPACS1*), linked to high levels of ethylene ( $\geq 10 \mu\text{l/kg f.w./hr}$ ) and B (0.8kb of *PPACS2*), linked to moderate levels of ethylene ( $0.5 \mu\text{l/kg f.w./hr} \sim 10 \mu\text{l/kg f.w./hr}$ ), when the total DNA was digested by *HindIII*. Four RFLP types (AB, Ab, aB, ab) were identified based on RFLPs using this gene diagnosis. The maximum ethylene level during fruit ripening in Japanese pear was proposed to be regulated mainly by the expression of these two ACC synthase genes and the action of A to mask that of B due to its much higher level of expression. Therefore, the RFLP types of AB and Ab show high levels and aB shows a moderate level of ethylene synthesis during fruit ripening. The gene diagnosis for ethylene production

using allele-specific PCR amplification and restriction enzyme digestion was developed. Moreover, the ethylene synthetic genotypes of Japanese pear cultivars commercially important and used in breeding were determined using the allele-specific PCR-based method to test cross lines. For example, the genotypes of 'Kosui', 'Shinsui', 'Chojuro', 'Yakumo', 'Cili', and 'Yali' were *aaBb*, *aaBB*, *aaBb*, *aaBb*, *AaBB* and *AABb*, respectively. This information is critical to the breeding program for selection of parents and useful for breeding strategies to improve storage ability in Japanese pear.

#### **Chapter 4 Isolation of fruit ripening related genes and possible involvement of ethylene in their expression**

Genes up-regulated during fruit ripening were identified to elucidate the molecular basis of fruit ripening in Japanese pear. Three up-regulated cDNAs containing a complete open reading frame were isolated using differential display techniques. The most interesting cDNA cloned was *JPRXYL*. Sequence comparisons indicated that the protein encoded by *JPRXYL* mRNA is a  $\beta$ -D-xylosidase. This is the first report on the cloning of a  $\beta$ -D-xylosidase gene from plants.

Thirty cDNA clones of genes corresponding to mRNAs up-regulated in fruit ripening of Japanese pear cv. Kikusui were obtained by differential screening of ripe fruit cDNA library. All of these cDNAs were sequenced and classified into 11 nonredundant groups after database searches.

The possible involvement of ethylene in the expression of up-regulated

genes isolated by differential display or differential screening was investigated. The accumulation of transcripts of 3 out of 14 genes was inhibited by 1-methylcyclopropene (MCP), an inhibitor of ethylene action. This suggests that the expression of these three genes is subject to the ethylene signal transduction pathway. The expression of *JPRXYL* was not affected by the MCP treatment. This indicated that *JPRXYL* is an ethylene-independent gene and that hemicellulose disassembly may be regulated by ethylene-independent processes.

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